Identification of Pax5 as a Target of MTA1 in B-Cell Lymphomas

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Abstract

Previously, we have shown that metastasis-associated protein 1 (MTA1) overexpression in transgenic mice was accompanied by high incidence of spontaneous B-cell lymphomas including diffuse large B-cell lymphomas (DLBCL). To understand the molecular basis of lymphoma in MTA1-transgenic (MTA1-TG) mice, we wished to identify a putative MTA1 target with a causal role in B-cell lymphogenesis. Using chromatin immunoprecipitation assays, we identified paired box gene 5 (Pax5), a molecule previously implicated in B-cell lymphogenesis, as a potential downstream effector of MTA1. Lymphomas from MTA1-TG mice also showed up-regulation of Pax5. We also found that MTA1 acetylated on Lys626 interacted with p300 histone acetyltransferase, and that acetylated MTA1 was recruited to the Pax5 promoter to stimulate Pax5 transcription. Global gene profiling identified down-regulation of a set of genes, including those downstream of Pax5 and directly implicated in the B-cell lymphogenesis. Significance of these murine studies was established by evidence showing a widespread up-regulation of both MTA1 and Pax5 in DLBCL from humans. These observations provide in vivo genetic evidence for a role of MTA1 in lymphomagenesis. [Cancer Res 2007;67(15):7132–8]

Introduction

B-cell malignancies are generally associated with specific chromosomal alterations that result in the oncogenic conversion of regulatory genes with roles in proliferation, apoptosis, and differentiation. In addition, these cancers show blockade of normal B-cell differentiation at specific developmental stages ranging from germinal center-like B cells to plasmacytic cells (1). Paired-box gene 5 (Pax5) is a B-cell–specific essential transcription factor with functions in the early B-cell development as well as late differentiation (1–5). Pax5 expression is developmentally regulated during B-cell maturation, starting from the earliest B-lineage precursor cells to mature B-cell stage, and lost on differentiation into plasma cells. Pax5 participates in a series of regulatory networks that control plasma cell differentiation by repressing the X-box binding protein 1 (XBP-1) transcription factor (6). The loss of Pax5 expression during differentiation relieves Pax5 repression of XBP-1, allowing terminal differentiation of B lymphocytes into plasma cells (7). Pax5 represents a common deregulated molecule in multiple B-cell malignancies including lymphoplasmacytic lymphoma, intravascular lymphoma, primary central nervous system lymphoma, and diffuse large B-cell lymphoma (DLBCL; refs. 8–12). In a significant number of these cancers, Pax5 has been reported to be either up-regulated or mutated due to aberrant hypermutation (9, 11). In lymphoplasmacytic lymphoma and a few cases of DLBCL, elevated expression of Pax5 is the result of chromosomal translocation that juxtaposes Pax5 to the immunoglobulin heavy chain (IgH) gene promoter (8, 12). However, literature recording Pax5 overexpression without translocation exist (8), indicating that alternative mechanisms could potentially play a role in inducing aberrant expression of Pax5 in B-cell malignancies. Thus, in spite of the widely acknowledged role of Pax5 in the pathogenesis of B-cell lymphomas, transcriptional regulator(s) of Pax5 expression with a role in the development of spontaneous B-cell lymphomas are not known. Here, we identified metastasis-associated protein 1 (MTA1) as a transcriptional regulator of Pax5. Further, Pax5 was up-regulated in B-cell lymphomas from MTA1-transgenic (MTA1-TG) mice as well as in human DLBCL and B-cell lymphoma cell lines, and correlated well with MTA1 expression.

Materials and Methods

Cell culture and antibodies. KIS-1 and SP53 cells were maintained in RPMI 1640 supplemented with 10% FCS. Antibodies against MTA1, Pax5, and histone deacetylase (HDAC)-2 were purchased from Santa Cruz Biotechnology.

Plasmid construction and mutagenesis. T7-tagged wild-type (WT) MTA1 full-length expression vector and generation of the Lys626 to alanine mutation of the MTA1 construct have been described elsewhere (13, 14). To clone the Pax5 regulatory region, DNA isolated from KIS-1 cells was amplified by PCR using the primers Pax5 enhancer-F and Pax5 enhancer-R to generate a 450-bp fragment and cloned into the PGL2-luc. The Pax5 promoter was cloned from DNA isolated from KIS-1 cells by PCR using the primers Pax5 promoter-F3 and Pax5 promoter-R2 to generate a 450-bp fragment and cloned into PGL2-luc. Primers used for reverse transcription-PCR (RT-PCR) are listed in Supplementary Table S1. Note: Supplementary data for this article are available at Cancer Research Online (http://cancersres.aacrjournals.org/).

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Affymetrix GeneChip expression analysis. The Mouse Genome 430 2.0 GeneChip contains 45,037 probe sets representing more than 34,000 named genes based on GenBank and/or RefSeq database sequences. cRNA synthesis, hybridization, and posthybridization staining were done as previously described (15, 16) using standard protocols as recommended by the manufacturer (Affymetrix). Genes identified as significantly differentially expressed between normal tissue and tumor samples were clustered by using TIGR Multi Experiment Viewer (TMEV).5

Pax5 promoter sequence analysis. A total of 1,386 tumor-associated transcripts were identified based on Affymetrix GeneChip analysis and targeted for promoter sequence analysis using TRANSFAC (17). The mouse genome assembly corresponding to Build 35 was downloaded from the UCSC genome browser.6 Each transcript sequence was mapped to the mouse genome using GMAP (18) and sim4 (19) as part of a PASA pipeline (20). Only those transcripts that mapped nearly perfectly to the genome, with misaligning at least 90% of their length and at least 95% identity with consensus splice sites at intron boundaries, were subjected to promoter analysis. The Pax5_01 and Pax5_02 matrices in the TRANSFAC transcription factor binding site library were used in the searches. Altogether, 218 Pax5 sites were identified (207 Pax5_01 sites and 11 Pax5_02 sites) in 211 promoters.

Statistical analysis. Groups were compared using two-tailed Student's t tests. P < 0.05 was considered significant.

Results

Recently, we reported that MTA1-TG mouse develop high incidence of spontaneous lymphomas. To understand the molecular basis of lymphoma in MTA1-TG mice, we wished to identify a putative MTA1 target with a causal role in B-cell lymphogenesis. While characterization of DLBCL in MTA1-TG mice was in progress, we were also attempting to identify potential direct chromatin targets of MTA1 in breast cancer cells. Our approach included the use of MTA1-specific, antibody-based double chromatin immunoprecipitation in MCF-7 cells. One of the MTA1 targets relevant in the context of lymphomas as a potential downstream effector of MTA1 was an enhancer sequence in the 7th intron of the paired box gene 5 (Pax5; Fig. 1A; Supplementary Table S2).

Because gene expression has been shown to be regulated by enhancers almost 100 kbp away from transcription start site (21) as well as by promoter elements, we proceeded to validate the cloned fragment as a bona fide target of MTA1 in vivo; primers were designed encompassing the cloned fragment as well as 150 bp on either side of the fragment for the chromatin immunoprecipitation assay. For initial validation studies in MCF-7 cells, chromatin immunoprecipitation experiments using either MTA1 antibody or no-antibody control indicated that MTA1 could be recruited to this 450-bp region in the Pax5 intron (Fig. 1B). Guided by these findings, we next conducted a series of studies to understand the regulation of Pax5 by MTA1 in B cells.

To investigate the role of MTA1 in the context of B cells, the chromatin immunoprecipitation assay with MTA1 antibody was done in a diffuse large-cell lymphoma cell line, KIS-1, widely used to study Pax5 biology (22). In KIS-1 cells, MTA1 was recruited onto the chromatin immunoprecipitation target sequence on treatment with either serum (both 1% and 10%) or interleukin-7 (IL-7), a ligand known to affect Pax5 expression in B cells (Fig. 1C). Further, depletion of MTA1 in KIS-1 cells using MTA1-specific siRNA (Fig. 1D) followed by chromatin immunoprecipitation assay with MTA1 antibody showed a clear decrease in occupancy of the Pax5 chromatin by MTA1. Because coregulators can interact with their target promoters at multiple sites both upstream and downstream of transcription initiation site (21), we next designed primers against 12 kb of the 5'-flanking sequence upstream of exon 1A of the Pax5 gene and repeated the chromatin immunoprecipitation assays in KIS-1 cells. We found that MTA1 in KIS-1 cells is also recruited to the Pax5 promoter region (~1,434 to ~663 bp) in addition to the enhancer region (Fig. 1E). To define the regulation of Pax5 by MTA1 in a cell line devoid of Pax5 translocation, we looked at MTA1 recruitment on both the promoter and enhancer

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5 http://www.tigr.org/softlab
6 http://hgdownload.cse.ucsc.edu/goldenPath/mm7/bigZips/
regions in another lymphoma cell line, SP53. MTA1 could be recruited to the chromatin immunoprecipitation pull-down fragment in the Pax5 on serum stimulation (Fig. 2A, left) and recruited to the promoter sequence under basal conditions (Fig. 2A, right, lane 1), and serum treatment increased MTA1 occupancy on the Pax5 promoter (Fig. 2A, right, lane 2), indicating that MTA1 recruitment to both the promoter and enhancer regions is specific and occurs in multiple cell lines. To understand whether interaction with the promoter or enhancer was important, MTA1 was depleted and Pax5 expression was evaluated. Pax5 gene expression was compromised by MTA1 knockdown (Fig. 2B), suggesting that the interaction of MTA1 with the Pax5 enhancer sequence may be more relevant for regulation of Pax5 gene expression. To show that the noted recruitment of MTA1 on the Pax5 gene fragment had a functional stimulatory effect on Pax5 expression, we cloned this sequence into a PGL2-luc reporter system (23). We found a dose-dependent increase in the activity of the PGL2-Pax5 reporter in response to MTA1 expression in HeLa cells, indicating that MTA1 functions as an activator in the context of this Pax5 enhancer sequence (Fig. 2C).

Because Pax5 controls plasma cell differentiation by repressing XBP-1 and because spleens from the MTA1-TG mice contained increased number of B cells, we next examined the status of XBP-1 and because spleens from the MTA1-TG mice contained significantly up-regulated in DLBCL tumors from MTA1-TG mice. Pax5 was also exhibited co-upregulation of both Pax5 (Fig. 3C) and MTA1 (Fig. 3D; Supplementary Table S3). MTA1 and Pax5 were found to be up-regulated in other B-cell and T-cell lymphomas (Supplementary Table S4).

Because MTA1 acts as an activator of Pax5 gene expression in the context of lymphocytes, we sought to dissect the potential mechanism. Recently, we identified an acetylation motif, GKXXP, in the COOH terminus of MTA1 and we generated a point mutant of MTA1 where lysine was substituted by alanine at amino acid position 626 (MTA1-K626A; ref. 14). Transfection of either MTA1-WT or MTA1-K626A with and without p300 indicated that mutation of MTA1 Lys626 results in the complete abrogation of the acetylation signal, indicating that MTA1 Lys626 in the GKSYP motif is the primary target for acetylation (14). Because MTA1 has been shown to interact with HDACs (13), we also examined the effect of MTA1 acetylation on HDAC binding. MTA1-WT interacted with HDACs in vivo whereas MTA1-K626A interacted to a greater extent with HDAC2 (Fig. 4A), suggesting differential recruitment of HDACs/p300 to MTA1-WT and MTA1 acetylation mutant. We next examined the influence of MTA1 acetylation on the recruitment of a transcriptionally active MTA1 complex to the Pax5 chromatin. KIS-1 cells were transfected with T7-MTA1-WT or T7-MTA1-K626A and chromatin immunoprecipitation analysis for recruitment of the transfected T7-MTA1 protein on Pax5 regulatory region was done using a T7 monoclonal antibody. Eluates from the first chromatin immunoprecipitation were reimmunoprecipitated with an antibody against polymerase II (Pol II), a marker of functionally active transcriptional complexes. We found that Pol II was recruited to the Pax5 promoter in serum-stimulated cells expressing MTA1-WT and not MTA1-K626A in spite of its binding to Pax5 promoter (Fig. 4B), suggesting the existence of productive MTA1/Pol II active complex in vivo and its recruitment to the Pax5 regulatory chromatin only with the WT-MTA1. To determine the functional consequence of MTA1 acetylation on Pax5 transcriptional activity, vectors expressing MTA1-WT or MTA1-K626A were cotransfected into HeLa cells along with PGL2-Pax5. We found that MTA1-K626A exhibited a significant reduced ability to induce transcriptional activity (Fig. 4C), whereas MTA1-WT activated PGL2-Pax5 luciferase as seen earlier.

**Figure 2.** A, chromatin immunoprecipitation assay was done in SPS3 cells using either enhancer- or promoter-specific primers for PCR. B, KIS-1 and SP53 cells were transfected with MTA1 or control siRNA for 72 h and cell extracts were blotted with indicated antibodies. C, 450-bp fragment of the Pax5 seventh intron was cloned into the PGL2-luc, and Pax5-transcriptional activity was estimated in HeLa cells. D, down-regulation of XBP-1 in B cells isolated from MTA1-TG mice. RT-PCR analysis of XBP-1, Pax5, c-Myc, and T7-MTA1 expression in B cells isolated from spleen of 6-wk-old MTA1-TG mice (n = 4) and WT mice (n = 4).
These findings suggest that MTA1 may represent an important modulator of Pax5 expression. To gain further insight into the mechanisms of MTA1-induced lymphomas, we carried out Affymetrix GeneChip Expression Analysis using the Mouse Genome 430 2.0 Gene Chip to investigate the differences in gene expression profiles between lymphomas observed in the MTA1-TG and control WT mouse tissues. A total of 5,177 genes were found to be differentially expressed between the WT spleen and spleen lymphoma tissue and 5,274 genes between the WT intestine and intestinal lymphoma tissue (Supplementary Fig. S1). We wanted to narrow our analysis to genes that followed the same pattern of expression between the intestine and spleen lymphomas as compared with the WT tissues (Fig. 5A; Supplementary Table S5). This was accomplished by applying a feature selection procedure to the gene expression data (16, 24), resulting in the identification of a core set of 1,068 genes, herein referred to as the lymphoma gene cluster. Genes contained in the lymphoma cluster were functionally classified into Gene Ontology categories using the bioinformatics application Expression Analysis Systematic Explorer and significance scores were calculated by carrying out 10,000 interactions (ref. 25; Supplementary Tables S6–S8). Inspection of the individual categories revealed that lymphomas had increased expression of genes previously implicated in lymphoma development (e.g., Ccl9, Lck, PARP, Pou2af1, and Cdk6; refs. 12, 26–28) and B-cell function (e.g., Pou2af1), increased expression of proliferative (e.g., Pes1, Edg3, and Cse1l) and antiapoptotic (e.g., Ywhag and Hells) genes not previously associated with lymphomas, and decreased expression of genes known to be repressed by Pax5 (e.g., Emp1, Pik3r1, Slc16a10, Serpinb1a, and C030038J10Rik; ref. 29; Fig. 5A). Next, we validated the differential expression of the genes in the lymphoma cluster by RT-PCR analysis of WT spleen and lymphomas arising in the spleen and intestine (Fig. 5B) and some by Northern blot analysis (Fig. 5C). These results suggest that MTA1 may promote...
lymphomas in our transgenic mouse model which, at least in part, may be mediated by up-regulation of Pax5, which in turn regulates the expression of a subset of genes within the lymphoma cluster.

Next, we identified 218 Pax5 sites in 211 gene promoters that might be affected in lymphomas using the TRANSFAC promoter sequence analysis tool (17). There was an even distribution of up-regulated and down-regulated genes containing a Pax5 site, and the changes in gene expression ranged from ~2-fold to >100-fold between normal and tumor samples (Supplementary Fig. S2; Supplementary Table S9). Of the 211 promoters with Pax5 binding sites, we validated by RT-PCR the differentially expression of 3 genes (Apex1, Msn, and Hank) which had a perfect Pax5 binding motif score, and further by Northern blot analysis. This analysis confirmed the repression of Hank and Apex and the up-regulation of Msn in lymphomas from the MTA1-TG mice as compared with tissues from the WT mice (Fig. 6A).

Next, we carried out chromatin immunoprecipitation analysis to verify the recruitment of Pax5 to selected target promoters of two down-regulated and two up-regulated genes selected from the Northern blot analysis. We next verified the modulation of Pax5 interactions to the selected target promoters in chromatin isolated from three murine lymphomas and three WT spleen tissues. Results indicated that Pax5 was recruited to the promoters of C030038J10Rik and Slc16a10 (Pax5 repressed genes) as well as that of Pou2af1 and Msn in the WT spleen (Fig. 6C, W1–W3). There was a significant increase in the recruitment of Pax5 to the target promoters in the transgenic lymphoma samples (Fig. 6C, T1–T3), indicating that the increased Pax5 expression observed in MTA1-TG mice (Fig. 3A) was also accompanied by a corresponding regulation of expression of the Pax5 target genes.

To delineate the role of signals in the recruitment of Pax5 to the four target genes validated above in the mouse samples, we examined the interactions of Pax5 with the selected target promoters using KIS-1 cells stimulated with serum or IL-7. Our results indicated that Pax5 was recruited to the promoters of C030038J10Rik and Slc16a10 and serum or IL-7 promotes derecruitment of Pax5 from these promoters (Fig. 6D, top). In contrast, Pax5 recruitment onto the promoters of the two Pax5 activated genes, Pou2af1 and Msn, was distinctly increased on stimulation with serum and IL-7 (Fig. 6D, bottom). These results confirmed that several MTA1-responsive genes are also direct targets of Pax5 and,
Thus, MTA1-induced changes in gene expression might, in part, be influenced by the up-regulation of Pax5 by MTA1.

Discussion

Pax5, a B-cell–specific essential transcription factor, functions in the early B-cell development as well as in late differentiation (1–5). At B-cell lineage, Pax5 fulfills a dual role of coactivator and repressor by activating B-cell–specific genes and repressing lineage-inappropriate genes (3). Pax5 activates target genes encoding essential components of (pre)B-cell receptor (BCR) signaling such as the signaling chain Igα (mb-1, CD79a), the stimulatory coreceptor CD19, and the central adaptor protein BLNK (3). Transactivation by Pax5 facilitates signal transduction from the pre-BCR and BCR, which constitute important checkpoints in B-cell development. Conversely, it was found that Pax5-dependent repression of the Csf1r (M-CSFR) and Notch1 genes renders committed B cells unresponsive to the myeloid cytokine M-CSF or to T-cell–inducing Notch1 ligands (3). Based on this repression of these two genes, it was hypothesized that the repression function of Pax5 may contribute to B-lineage commitment by shutting down inappropriate signaling systems. To further strengthen this notion, recently, 110 Pax5-repressed genes were identified by cDNA microarray analysis, which provided important insights into the repression function of Pax5 during B lymphopoiesis (29). Pax5 is a deregulated molecule in multiple B-cell malignancies including lymphoplasmacytic lymphoma, intraocular lymphoma, primary central nervous system lymphoma, and DLBCL (8–12).

Although numbers of studies have elucidated the role of Pax5 function in B-cell development, the physiologic upstream regulators of Pax5 are not well studied. Our results show for the first time that Pax5 chromatin is a physiologic target of MTA1 and that MTA1 is a coactivator of Pax5 expression and functions. Because deregulation of Pax5 and, consequently, its effector pathways is believed to contribute to the development of B-cell lymphomas and because we have now shown that MTA1, a chromatin modifier and new identified coactivator of Pax5, is commonly up-regulated in B-cell lymphomas, the dysfunction of MTA1 regulation of Pax5 in B cells reported here may constitute an important pathway in the pathogenesis of B-cell lymphomas. Global gene expression analysis has identified a core set of lymphoma-associated genes. Some of the down-regulated Pax5 site–containing genes in MTA1-TG mice...
are known targets for repression by Pax5, and a number of the up-regulated Pax5 site–containing genes have been implicated with lymphoma development. Overall, these findings suggest a model wherein MTA1-mediated deregulation of Pax5 expression might block differentiation and stimulate proliferation of B cells and, consequently, contribute to enhanced cell survival, clonal expansion of B cells, and development/maintenance of B-cell lymphomas.

References


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Correction: Identification of Pax5 as a Target of MTA1 in B-cell Lymphomas

In this article (Cancer Res 2007;67:7132–8), which appeared in the August 1, 2007, issue of Cancer Research (1), there are errors in the input lanes in Fig. 2A and vinculin lane in Fig. 2B, and duplication of the actin mRNA panel in Fig. 3A from the accompanying article by Bagheri-Yarmand and colleagues (2). The authors have replaced Figs. 2A, 2B, and 3A using data from repeat experiments. In addition, four duplicated MTA1 immunohistochemistry panels from the accompanying article by Bagheri-Yarmand and colleagues reused in Fig. 3D have been removed. There is no change in the legend or text for Fig. 2. In the text on page 7134, the last sentence in the first column should read, “Similarly, Pax5 immunohistochemistry of the human DLBCLs also showed strong nuclear Pax5 staining (+3 staining) in 52 of 76 (68.4%) cases (Fig. 3C).” These changes do not change the original scientific conclusions, and the validity of the findings remains the same.

The legend for Fig. 3 should read as follows:

Pax5 expression in MTA1-TG lymphomas. A, RT-PCR analysis of Pax5 expression in MTA1-TG lymphoma tumors. B, representative tumor samples isolated from spleen, gastrointestinal tract (GI), and control tissue (spleen) were blotted with an anti-Pax5 antibody. C, expression of MTA1 in human DLBCL samples.

The corrected figures appear below. The authors regret these errors.
References


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