Epigenetic Regulation of CXCR4 Expression by the Ocular Microenvironment

Haochuan Li, Jerry Y. Niederkorn, Leila Sadegh, Jessamee Mellon, and Peter W. Chen

PURPOSE. Expression of the chemokine receptor CXCR4 by tumors is associated with metastatic migration and invasion of tumor cells. The importance of CXCR4 expression by uveal melanomas in metastasis to the liver was recently demonstrated when injection of CXCR4-negative uveal melanoma cells into mice resulted in reduced liver metastasis compared with CXCR4-positive uveal melanoma cells. Factors in the eye can induce downregulation of genes by epigenetic mechanisms. This study examined whether epigenetic regulation by the ocular environment induced downregulation of CXCR4 expression.

METHODS. LS174T colon cancer cells were injected in the anterior chamber (AC), subcutaneously (SC), or in the spleen capsule to induce liver metastasis in immune-deficient mice. CXCR4 gene transcription was analyzed by RT-PCR, and protein expression was determined by flow cytometry. Methyltransferase and histone deacetylase activities were determined by ELISA. Treatment with either 5-Aza-2-deoxycytidine (5-Aza) or trichostatin A (TSA) was used to induce demethylation or inhibit histone deacetylases, respectively.

RESULTS. AC-derived LS174T cells showed lower CXCR4 gene expression compared with SC-, liver-derived, or wild-type tumor cells. AC-derived LS174T tumor cells expressed methyltransferase activity compared with SC-, liver-derived, and wild-type tumor cells. Deacetylase activity was elevated in AC-derived LS174T tumor cells compared with SC-derived, liver-derived, and wild-type tumor cells. Treatment of AC-derived LS174T tumor cells with 5-Aza upregulated CXCR4 expression. TSA treatment did not restore CXCR4 expression.

CONCLUSIONS. These studies demonstrate that ocular microenvironment factors induce methylation and downregulation of tumor CXCR4 expression. (*Invest Ophthalmol Vis Sci.* 2013; 54:234–243) DOI:10.1167/iovs.12-10643

Chemokines are small protein molecules that play a critical role in development and host defense mechanisms by promoting directional migration and activation of immune cells.¹ They provide signals to direct lymphocyte trafficking by inducing cellular adhesion and transmigration across endothelial cell membranes. Hematopoietic and nonhematopoietic cells produce chemokines constitutively, or production can be induced by injury, or other proinflammatory stimuli.² As such, chemokines are detected in several proinflammatory human diseases, including irritable bowel disease, rheumatoid arthritis, and HIV, and in experimental animal models, including experimental autoimmune encephalomyelitis (EAE) that resembles multiple sclerosis and airway hyperreactivity models that resembles asthma.^{3–6}

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Paget's "seed and soil" hypothesis predicted that tumor cells successfully metastasized to locations within the host that were favorable for tumor growth.⁷ It was appealing to hypothesize that constitutive tumor expression of factors like chemokine receptors could facilitate metastatic migration of tumor cells to distant sites in an organ-specific manner. Studies have demonstrated that tumor expression of chemokine receptors promotes tumor cell dissemination at many steps of metastasis, which include migration to distant organ-specific locations, the adherence of tumor cells to vascular endothelium and extravasation from blood vessels, angiogenesis, and protection from the anti-tumor immune responses.^{8,9} Chemokine receptors and their respective ligands, notably the CXCR4/CXCL12 combination, have been implicated in directional migration of many cancers to specific organs.¹⁰ Muller et al. demonstrated that breast cancer progression followed a distinct pattern of metastasis to the lymph nodes, bone marrow, lung, and liver.¹¹ Breast cancer cells express the chemokine receptors CXCR4 and CCR7. These chemokine receptors have been correlated to the metastatic spread of breast cancer to lymph nodes, lungs, and the liver, which constitutively express high levels of their associated ligands CXCL12—also known as stromal cell-derived factor 1 (SDF-1)—and CCL21, respectively, in the target organs. Similarly, studies by Zeelenberg et al. showed that metastatic spread of CXCR4-positive colorectal cancer to the liver was due to the high liver expression of CXCL12.¹² Uveal melanomas, the most common form of ocular tumors in adults, preferentially metastasize to the liver in 95% of patients with this disease.^{13,14} Recently, we demonstrated that uveal melanoma expression of CXCR4 and CCR7 provided directional migration of these tumor cells to the liver.¹⁵ Moreover, inhibition of uveal melanoma expression of CXCR4 by siRNA transfection diminished the invasive properties of uveal melanoma cells and reduced the number of metastasis to the liver in an experimental animal model.¹⁶ Therefore, the downregulation of CXCR4 expression by siRNA transfection may be used as a therapeutic strategy to prevent metastasis of uveal melanoma to the liver. However, the use of siRNA for antitumor therapy faces many challenges due to instability of siRNA in the circulation, poor intracellular incorporation, and potential induction of nonspecific immune responses.¹⁷

Recent studies have focused on the role of epigenetic gene regulation in the pathophysiology of cancer. Genomic modifications have been found frequently in cancer due to inherent genetic instability and almost always result in a permanent

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Supported by NIH NEI Grants EY017198 (PWC); CA30276 (JYN) and EY020799 (Core Grant); and an unrestricted grant from Research to Prevent Blindness.

Submitted for publication July 25, 2012; revised November 12, 2012; accepted November 17, 2012.

Disclosure: H. Li, None; J.Y. Niederkorn, None; L. Sadegh, None; J. Mellon, None; P.W. Chen, None

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change in cell phenotype. By contrast, epigenetic alterations in cancer are reversible, and indeed, epigenetic aberrations have been identified in many tumors, including breast cancer, pancreatic cancer, and skin melanomas.^{18,19} It is interesting to note that CXCR4 expression in human skin melanoma was upregulated when these cells were treated with either a demethylating agent (5-Aza 2-deoxycytidine [5-Aza]) or a histone deacetylase (HDAC) inhibitor (trichostatin A [TSA]), suggesting that an epigenetic mechanism regulates expression of CXCR4 by these tumors.²⁰ Data from our recent studies suggest that factors in the aqueous humor found in the anterior chamber of the eye induces epigenetic gene regulation in an experimental murine ocular tumor model (Chen PW, unpublished observations, 2011).²¹ In this study, we used the CXCR4⁺ LS174T colon cancer cells to determine whether CXCR4 gene expression by these cells could be downregulated by epigenetic gene regulation and whether differences in CXCR4 expression were due to either epigenetic methylation and/or chromatin remodeling. Since uveal melanomas have been exposed to the ocular environment that may induce epigenetic regulation, we used the human LS174T colon adenocarcinoma cell line for these studies since they originated in an organ that, to our knowledge, does not induce epigenetic influence, and because LS174T cells constitutively express the chemokine receptor CXCR4.

MATERIALS AND METHODS

Mice

Balb/c NOD-SCID mice were used to establish organ-specific tumor cell lines. All animal studies were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center, and the animals were housed and cared for in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Cell Lines

LS174T is a human colon adenocarcinoma cell line obtained from American Type Culture Collection (Manassas, VA). LS174T cells were expanded in culture, tested for mycoplasma, and aliquoted for cryogenic storage. Establishment of organ-specific LS174T tumor cells is described below. All cell lines were cultured in complete RPMI 1640 (JRH Biosciences, Lenexa, KS) and supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cell lines were cultured for no more than 10 consecutive serial passages before they were discarded and a new aliquot of cells were thawed and used for experiments. Generation of organ-specific LS174T tumor cells is described below.

Anterior Chamber Injections

Anesthetized Balb/c NOD SCID mice were given an anterior chamber injection of LS174T tumor cells (1×10^5 cells/6 μ L) using a glass micropipette fitted onto a 0.1-mL Hamilton syringe (Hamilton Co., Reno, NV). Tumor-bearing eyes were enucleated 18 days postinjection and aseptically explanted tumor cells were cultured in complete RPMI 1640 (JRH Biosciences). The anterior chamber-derived cell line was designated "LS174T-AC."

Intrasplenic Injections

Liver metastases from LS174T tumor cells were generated in Balb/c NOD-SCID mice. LS174T cells (1×10^6 cells/100 mL) were transplanted under the spleen capsule. Mice underwent necropsy 35 days postinjection, and metastatic foci were aseptically isolated from the livers and cultured in complete RPMI 1640 (JRH Biosciences). The metastatic cell line was designated "LS174T-LM."

Cecal Wall Injections

Cecal-derived LS174T tumor cells were generated in Balb/c NOD-SCID mice. LS174T cells (1×10^6 cells/100 mL) were injected into the cecal wall. Mice underwent necropsy 30 days postinjection, and tumors were aseptically isolated from the cecum and cultured in complete RPMI 1640 (JRH Biosciences). The cecal-derived cell line was designated "LS174T-CEC."

Subcutaneous Injections

Subcutaneously derived LS174T tumor cells were generated in Balb/c NOD-SCID mice. LS174T cells (5×10^6 cells/100 mL) were injected subcutaneously in NOD-SCID mice. Mice underwent necropsy 12 days postinjection, and tumors were aseptically isolated from tumor-bearing mice and cultured in complete RPMI 1640 (JRH Biosciences). The subcutaneously derived cell line was designated "LS174T-SC."

Flow Cytometric Analysis

Surface expression of CXCR4 was assessed by flow cytometry. After washing 3 $\times$ with Hanks' balanced salt solution (HBSS), cells were maintained in HBSS containing 0.3% BSA. Cells (1×10^6) were incubated with 1 μ g/mL of purified anti-human CXCR4-specific antibody (eBioscience, San Diego, CA) for 30 minutes at 4°C. Cells were washed 3 $\times$ with HBSS containing 0.3% BSA, resuspended in 0.5 mL PBS, and assessed for fluorescence in a flow cytometer (FACScan Flow Cytometer; BD Biosciences, Palo Alto, CA). The data were analyzed using data analysis software (CellQuest, version 3.1f; BD Biosciences).

Reverse Transcription PCR

Total RNA from various tumor cell lines was isolated (RNAqueous; Ambion, Austin, TX) and treated with DNase I (0.2 U/ μ L; Ambion) to remove possible DNA contamination. Total RNA was converted into first-strand cDNA using a PCR array kit (RT² First Strand Kit; Qiagen, Valencia, CA) according to the manufacturer's conditions. Primers for human CXCR4 were: 5'-AAT CTT CCT GCC CAC CAT CT-3' and 5'-GAC GCC AAC ATA GAC CAC CT-3' (Qiagen). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as an internal control (Qiagen). Reverse transcription and PCR were performed on a thermal cycler (MJ Systems PTC-200 Thermocycler; Bio-Rad, Hercules, CA) using the following conditions: 95°C, 5 minutes; 28(95°C, 30 seconds; 60°C, 30 seconds; 72°C, 30 seconds); 72°C, 5 minutes. PCR products were separated on a 2% agarose gel by electrophoresis and imaged using an imaging system (ChemiDoc XRS Imager; Bio-Rad).

Bisulfite Conversion

DNA from LS174T, LS174T-AC, LS174T-SC, LS174T-CEC, and LS174T-LM cell lines was isolated using a monophasic reagent (DNA Stat-60; Tel-Test Inc., Friendswood, TX), and unmethylated cytosines were converted to uracils using a bisulfite conversion kit (EpiTect Bisulfite Kit; Qiagen) according to manufacturer's instructions. Bisulfite conversion of LS174T-CEC DNA was unsuccessful on multiple occasions and resulted in excessive DNA degradation. Therefore, we were not able to test LS174T-CEC for promoter CpG methylation described below.

Methylation-Specific PCR

Bisulfite-converted DNA from LS174T, LS174T-AC, LS174T-SC, and LS174T-LM cell lines were analyzed using methylation-specific PCR to assess the methylation status of CpG islands within the CXCR4 promoter²² using the following primers: Unmethylated CXCR4 primer: Fwd: 5'-TTA TTT GTT TTT AGG TGT ATT TTG T-3'; Rev: 5'-AAA CCC TAC TAT TTA CAA ATA ATC AAT-3'; methylated CXCR4 primer: Fwd:

5'-TTT TAT TTG TTT TTA GGC GTA TTT-3'; Rev: 5'-ACC CTA CTA TTT ACG AAT AAT CGA T-3'. PCR was performed on a thermal cycler (Bio-Rad) using the following conditions: 95°C, 5 minutes; 25(95°C, 30 seconds; 60°C, 30 seconds; 72°C, 30 seconds); 72°C, 5 minutes. PCR products were separated on a 1.5% agarose gel by electrophoresis and imaged using an imaging system (Bio-Rad). Band densitometry was performed on a high-resolution PCR image using a Java-based image processing program (ImageJ64, version 1.46r for Mac; <http://imagej.nih.gov/ij/>, in the public domain).

Treatment of Cells with Demethylating Agents and HDAC Inhibitors

Tumor cells (2×10^6) were cultured in complete RPMI 1640 (JRH Biosciences) containing either 5-Aza (0.5 μ M, 1.0 μ M, or 1.5 μ M; Sigma, St. Louis, MO) or TSA (100 nM, 250 nM, or 500 nM; Sigma) for 48 hours. Treated cell lines were allowed to recover and used for assays within six passages. Treatment of LS174T-CEC and LS174T-LM tumor cells with either agent was toxic at any concentration tested.

DNA Methyltransferase Assay

Nuclear extracts from wild-type, cecal wall-derived, anterior chamber-derived, skin-derived, and liver-derived LS174T tumor cells were isolated, expanded, and assessed for de novo DNA methyltransferase activity using a DNA methyltransferase activity kit (Epigentek, Farmingdale, NY) according to the manufacturer's protocol.

H3-K9 Methyl Histone Quantification

Cell lysates from various LS174T cell lines were assessed for monomethylation, dimethylation, or trimethylation of histone H3-K9 using a histone methylation quantification kit (EpiQuik Global Pan-Methyl Histone H3K9 Quantification Kit; Epigentek) according to the manufacturer's protocol.

HDAC Activity Assay

Nuclear extracts from various LS174T cell lines were assessed for HDAC activity using an HDAC activity assay kit (EpiQuik HDAC Activity/Inhibition Assay Kit; Epigentek) according to the manufacturer's protocol.

Statistics

The Student's *t*-test was used to determine significance in differences between experimental groups and controls. Differences were considered significant if the *P* values were <0.05 .

RESULTS

Lowered Expression of CXCR4 Expression in Eye-Derived LS174T Tumor Cells

Transcription and protein expression of CXCR4 on wild-type LS174T cells; eye-derived LS174T cells (LS174T-AC); skin-derived LS174T cells (LS174T-SC); liver-derived LS174T cells (LS174T-LM); and cecal wall-derived LS174T cells (LS174T-CEC) by FACS analysis and by reverse transcription PCR. LS174T cells transcribed the CXCR4 gene and expressed CXCR4 protein (Figs. 1A, 1B, respectively). By contrast, eye-derived LS174T-AC cells demonstrated a profound diminution of CXCR4 gene transcription and a 70% reduction in CXCR4 protein expression. CXCR4 gene and protein expression by LS174T cells derived from the skin, liver, and cecal wall expressed CXCR4 at roughly the same levels as wild-type LS174T cells. Mean channel fluorescence values for LS174T-AC

cells was also lower when compared with LS174T or LS174T cells derived from other sites (Fig. 1C).

Expression of CXCR4 by LS174T-AC Cells after Treatment with 5-Aza

Previous experiments have suggested that factors in the ocular environment upregulates expression of de novo methyltransferases that induce epigenetic regulation by gene methylation resulting in downregulation of gene expression (Chen PW, unpublished results, 2011). We hypothesized that downregulation of CXCR4 expression by LS174T-AC tumor cells was due to methylation of the CXCR4 gene. Accordingly, wild-type LS174T and LS174T-SC and LS174T-AC tumor cell derivatives were treated with the demethylating agent 5-Aza at various concentrations (0.5 μ M, 1 μ M, and 1.5 μ M) for 48 hours, and the expression of CXCR4 following 5-Aza treatment was determined by flow cytometry. CXCR4 expression was unchanged when wild-type LS174T cells and LS174T-SC cells were treated with 5-Aza (Fig. 2). By contrast, CXCR4 expression of LS174T-AC was upregulated after treatment with 1 μ M or 1.5 μ M of 5-Aza, suggesting that downregulation of CXCR4 in LS174T-AC cells was due to methylation of the CXCR4 gene.

DNA Methyltransferase Activity in LS174T-AC Tumor Cells

Downregulation in genomic expression by epigenetic methylation corresponds with the presence of de novo DNA methyltransferases that can methylate previously unmethylated CpG islands. As such, we investigated the expression of de novo methyltransferases by LS174T-AC, LS174T-SC, LS174T-CEC, LS174T-LM, and wild-type LS174T tumor cells. Expression of de novo methyltransferase by LS175T-SC, LS174T-CEC, and LS174T-LM tumor cells was similar to expression of de novo methyltransferases by the wild-type LS174T cells (Fig. 3). By contrast, de novo methyltransferases were significantly elevated in LS174T-AC cells when compared with the wild-type tumor cells.

CXCR4 Promoter Methylation of LS174T-AC Tumor Cells

Methylation of CpG islands within the gene promoter region specific genes can downregulate gene expression. We analyzed CpG methylation of four CpG islands within the CXCR4 gene promoter using methylation-specific PCR after bisulfite conversion of DNA. There was no change in the methylation status of three out of four CpG islands within the CXCR4 promoter region between wild-type LS174T cells and LS174T-AC, LS174T-SC, and LS174T-LM cells (data not shown). However, one CpG island in the promoter region of CXCR4, (~2660 bp–2671 bp) was partially methylated in LS174T, LS174T-SC, and LS174T-LM cell lines, but the same CpG island was almost completely methylated in LS174T-AC cells (Fig. 4). Next, we compared densitometry values between bisulfite-treated LS174T cells, and LS174T-AC, LS174T-SC, and LS174T-LM cell-PCR products from amplified with either unmethylated or methylated-specific primers. Image densitometry of the PCR products demonstrated that there was a significantly less expression of the unmethylated CpG island product by LS174T-AC cells compared with the same unmethylated PCR product by wild-type LS174T tumor cells (0.220 relative units vs. 1.048 units, respectively).

HDAC Activity of LS174T-AC Tumor Cells

Deacetylation of the H3 histone N-terminal tails is the predominant mechanism that results in the condensation of chromatin

FIGURE 1. Downregulation of CXCR4 by anterior chamber-derived LS174T cells. Wild-type, cecal wall-derived, anterior chamber-derived, skin-derived, and liver-derived LS174T tumor cells were isolated, expanded, and assessed for CXCR4 expression. (A) Total RNA was isolated from various cell lines and CXCR4 gene expression assessed by RT-PCR. (B) Cells were analyzed for CXCR4 expression by flow cytometry. (C) Mean channel fluorescence of cells labeled with secondary, isotype, or anti-CXCR4 antibody. Labeling of cells with isotype antibody or secondary antibody alone were used as background controls. * $P < 0.05$. Results of (A) are representative of three independent experiments. Results of (B, C) represent combined values from three independent experiments.

and the silencing of genes within that particular chromatin region. It was plausible that downregulation of CXCR4 expression by LS174T-AC tumor cells was due to increased histone deacetylase activity and silencing of the gene by hypoacetylation of the CXCR4 gene-associated chromatin. Accordingly, we prepared nuclear lysates from LS174T-AC, LS174T-SC, LS174T-CEC, LS174T-LM, and wild-type LS174T tumor cells and tested them for histone deacetylase activity. Expression of histone deacetylases by LS174T-SC, LS174T-CEC, and LS174T-LM tumor cells was similar to expression of de novo methyltransferases by the wild-type LS174T cells (Fig. 5). By contrast, the histone deacetylase activity was significantly upregulated by LS174T-AC cells when compared with the wild-type tumor cells.

Expression of Histone-3 Lysine-9 (H3-K9) Methylation by Eye-Derived LS174T-AC Cells

Methylation of lysine residues in the H3 N-terminal tails is another posttranslational modification that is involved in epigenetic gene regulation. We were particularly interested in methylation of H3-K9 since either monomethylation, dimethylation, and/or trimethylation of K9 has been associated with transcriptional repression of expression in human cancer cells.²³ Cell lysates from LS174T-AC, LS174T-SC, LS174T-CEC, LS174T-LM, and wild-type LS174T tumor cells and tested for mono- (H3-K9Me1), di- (H3-K9Me2), and trimethylation (H3-K9Me3) at the H3-K9 residue. H3-K9Me1, H3-K9Me2, and H3-

FIGURE 2. Expression of CXCR4 by 5-Aza 2-deoxycytidine (5-Aza 2-dC)-treated LS174T tumor cells. Wild-type, anterior chamber-derived, and skin-derived LS174T tumor cells were treated with various concentrations of 5-Aza for 48 hours. CXCR4 expression was determined by flow cytometry using CXCR4-specific antibody. All cell lines were also labeled with IgG isotype antibody or secondary antibody alone as controls (data not shown). $*P < 0.014$. Results represent combined values from four independent experiments.

FIGURE 3. Expression of de novo methyltransferases by wild-type and tissue-derived LS174T tumor cells. Wild-type, cecal wall-derived, anterior chamber-derived, skin-derived, and liver-derived LS174T tumor cells were cultured and used to prepare nuclear extracts. Samples were used in a fluorescence-based DNA methyltransferase assay kit according to manufacturer's instructions. $*P < 0.05$. Results represent combined values from three independent experiments.

FIGURE 4. Methylation of the CXCR4 promoter region in LS174T-AC tumor cells. DNA from wild-type, anterior chamber-derived, skin-derived, and liver-derived LS174T tumor cells was isolated and converted using a bisulfite conversion kit. Bisulfite-treated DNA was analyzed for CXCR4 expression by PCR using specific CXCR4 primers corresponding to methylated and unmethylated sequences. GAPDH expression was used as an internal control. Results are representative of two independent experiments. Densitometry was performed using a Java-based image processing program. Relative units were calculated as U/M and values were also compared between the various cell lines.

K9Me3 by LS175T-SC, LS174T-CEC, and LS174T-LM tumor cells was 2% to 5% higher than H3-K9 methylation levels in LS174T wild-type cells (Figs. 6A-C). Expression of H3-K9Me1 in LS174T-AC cells was also approximately 6% higher than H3-K9Me1 expression by LS174T-CEC, LS174T-SC, LS174T-LM, or wild-type LS174T cells (Fig. 6A). By contrast, expression of H3-K9Me2 and H3-K9Me3 by LS174T-AC cells was 16% and 21% higher, respectively, than expression by the wild-type LS174T cells, and demonstrated higher expression than LS174T-CEC, LS174T-SC, and LS174T-LM cells, suggesting that differential H3-K9 methylation occurs in the eye-derived tumor cells (Figs. 6B, 6C).

Expression of CXCR4 by LS174T-AC Cells after Treatment with TSA

HDAC inhibitors such as trichostatin A prevent the removal of acetyl groups from the N-terminal of histone tails, which promotes the formation of euchromatin and active gene transcription.²⁴ Since CXCR4 expression was downregulated in LS174T-AC cells, we wanted to determine whether treatment with TSA could restore CXCR4 expression in these cells. Accordingly, wild-type LS174T, LS175T-SC, and LS174T-AC tumor cells were treated with TSA at various concentrations (100 nM, 250 nM, and 500 nM) for 48 hours, and the expression of CXCR4 following TSA treatment was determined by flow cytometry. Expression of CXCR4 by TSA-treated wild-type LS174T and LS174T-SC cells was similar to the untreated control cells (Fig. 7). In addition, the expression of CXCR4 by LS174T-AC cells was also unchanged by treatment with increasing concentrations of TSA. Increased concentrations

of TSA (750 nM and 1000 nM) were toxic to all LS174T cell lines (data not shown).

DISCUSSION

Tumor cell plasticity plays a critical role in the pathogenesis of tumor progression by giving cancers the ability to metastasize to distant organs. This characteristic endows tumors with the ability to adopt mechanisms used for trafficking normal cells. As such, tumors can respond to chemokines by expressing chemokine receptors, which closely resemble mechanisms used in lymphocyte trafficking and allows them to migrate, invade, and metastasize in an organ-specific manner. To date, 19 chemokine receptors have been identified and characterized, and the one of the receptors most closely linked to metastasis is CXCR4.^{1,10}

This chemokine receptor is expressed on 23 different types of tumors and is associated with metastasis to the lymph nodes, bone marrow, skin, lungs, and the liver where its ligand, CXCL12, is highly expressed.¹⁰ While CXCR4 expression appears to play a critical role in metastasis, the mechanisms regulating the expression of this chemokine receptor is still being investigated.

Recently, there has been a great interest in elucidating the role of epigenetic gene regulation and its potential impact in tumor progression. Epigenetic regulation can dictate gene transcription globally through chromatin remodeling, or locally by DNA methylation. Chromatin remodeling is achieved by the actions of histone deacetylases that promote chromatin condensation into facultative heterochromatin and histone acetylases that facilitate chromatin relaxation and the forma-

FIGURE 5. HDAC activity by tissue-derived LS174T tumor cells. Wild-type, cecal wall-derived, anterior chamber-derived, skin-derived, and liver-derived LS174T tumor cells were cultured and used to prepare nuclear extracts. Samples were used in a fluorescence-based histone deacetylase assay kit according to manufacturer's instructions. HDAC activity was normalized to wild-type LS174T tumor cells. * $P < 0.05$. Results represent combined values from four independent experiments.

tion of euchromatin that can undergo active gene transcription.²² Upregulation of de novo DNA methyltransferases suppresses gene transcription by methylating cytosine-rich regions called CpG islands located within the gene promoter regions.^{22,25,26} Together, these two epigenetic mechanisms have been recognized as important pathways in tumor development and metastasis. We recently reported that

immunized mice against P815 murine mastocytoma cells could reject a subcutaneous tumor challenge of wild-type P815 tumor cells, but were unable to reject P815 tumor cells that were isolated from the anterior chamber of the eye.²⁷ Treatment of the eye-derived P815 cells with 5-Aza reversed the escape phenotype and the treated cells were rejected in P815 immunized mice. We determined that factors in the

FIGURE 6. Methylation of histone 3, lysine 9 (H3-K9) by tissue-derived LS174T tumor cells. Cell lysates were prepared from wild-type, cecal wall-derived, anterior chamber-derived, skin-derived, and liver-derived LS174T tumor cells and used to assess H3-K9. (A) Monomethylation. (B) Dimethylation. (C) Trimethylation in a colorimetric H3-K9 methylation quantitation assay kit. H3-K9 methylation was normalized to H3-K9 methylation in wild-type LS175T cells. * $P < 0.05$. NS, not significant. Results represent combined values from two independent experiments.

FIGURE 7. Expression of CXCR4 by TSA-treated LS174T tumor cells. Wild-type, anterior chamber-derived, and skin-derived LS174T tumor cells were treated with various concentrations of TSA for 48 hours. CXCR4 expression was determined by flow cytometry using CXCR4-specific antibody. All cell lines were also labeled with IgG isotype antibody or secondary antibody alone as controls (data not shown). Results represent combined values from three independent experiments.

aqueous humor, primarily TGF β , upregulated expression of de novo methyltransferases and histone deacetylases in tumor cells that were injected into the anterior chamber (Chen PW, unpublished data, 2011). Cells that were injected into the eye experienced increased global DNA methylation and histone deacetylation. Interestingly, studies by Mori et al. demonstrated that treating malignant cutaneous melanoma cells with either the demethylating agent 5-Aza or TSA upregulated expression of CXCR4 and enhanced cell migration.²⁰ In this study, we demonstrate that CXCR4⁺ LS174T colon cancer cells downregulate CXCR4 gene transcription and receptor expression when these cells are introduced into the eye. Expression of CXCR4 by CXCR4⁺ LS174T cells injected subcutaneously into the skin, the cecal wall, or intrasplenically to induce liver metastasis remained unchanged. Treatment of the LS174T-AC tumor cells with demethylating agent 5-Aza restored expression of CXCR4 by the eye-derived tumor cells but did not affect CXCR4 expression in the LS174T-SC, -CEC, or -LM tumor cells. Our results suggest that suppression of CXCR4 expression is epigenetically regulated by methylation of the CXCR4 gene. Studies by Bogani et al. demonstrated that suppression of CXCR4 expression by CD34⁺ cells from patients with primary myelofibrosis was due to hypermethylation of a CpG island in the CXCR4 gene promoter, and treatment of the CD34⁺ cells with 5-Aza restored expression of CXCR4 and showed improved migration in response to CXCL12.²⁸ Our findings are in congruence with these data since we demonstrate an increase in DNA methyltransferases in the LS174T-AC tumor cells, which is absent in the wild-type and also the LS174T cell lines derived from the different organs. Moreover, we determined that the CXCR4 promoter region of LS174T-SC and LS174T-LM showed both unmethylated and methylated CpG motifs in a pattern similar to the wild-type LS174T cells, whereas, LS174T-AC cells demonstrated almost complete methylation within a CpG island within the CXCR4 promoter. A possible reason why the parent LS174T cells and the LS174T cells derived from the skin and liver expressed both

unmethylated and methylated sites in the CXCR4 promoter could be due to heterogeneity of the cells within the tumor. Since the skin and liver cannot induce epigenetic changes through methylation, we observed both unmethylated and methylated CXCR4 in these cell lines. By contrast, eye-derived LS174T cells undergo methylation within the ocular environment, which results in methylation of unmethylated CpG regions in CXCR4 promoter. It is interesting to note that we also examined other CpG islands within the CXCR4 promoter by bisulfite conversion and PCR, and we did not observe differential expression of unmethylated and methylated CXCR4 PCR products (data not shown), suggesting that methylation of one CXCR4 CpG island is sufficient to downregulate expression of this gene in anterior chamber-derived LS174T cells. Together, these results indicate that factors in the ocular environment induce gene-specific methylation that is sufficient for the epigenetic downregulation of CXCR4 expression. The initial characterization of the mechanisms responsible for epigenetic downregulation are discussed below and also merit future investigation.

We also investigated whether downregulation of CXCR4 expression by LS174T-AC cells was potentially due to chromatin remodeling. Our present findings demonstrate that histone deacetylase activity is enhanced in LS174T-AC cells, but this activity remains unchanged in LS174T tumor cells derived from other organs. Interestingly, treatment of LS174T with the histone deacetylase inhibitor TSA did not restore CXCR4 expression by LS174T-AC cells. One explanation may be that chromatin condensation due to histone deacetylation is irreversible. The binding of histone tails to the transcriptionally silent condensed chromatin by sirtuin proteins such as SIRT1 inhibits chromatin relaxation by acetylation.²⁹ Another possible explanation could be that histone deacetylases do not initiate chromatin remodeling in the region surrounding the CXCR4 gene and that suppression of CXCR4 expression is dependent on methylation of CpG islands on the CXCR4 gene promoter region as described above. Our data suggests that the

latter possibility is the more likely scenario since treatment with the demethylating agent 5-Aza restored CXCR4 expression by LS174T-AC cells. It should be noted that TSA only represents one class of histone deacetylase inhibitor and that it may be possible that treatment of LS174T-AC cells with other classes of histone deacetylase inhibitors may restore CXCR4 expression.²⁴

Several lysine residues (K) on the H3 histone act as epigenetic marks that are susceptible to methylation by histone methyltransferases resulting in epigenetic gene silencing.³⁰ Moreover, lysine residues can be methylated with up to three methyl groups, and the different levels of methylation can be associated with unique genomic locations and functions.³¹ For example, H3-K9 mono- and demethylation is localized to euchromatin, whereas H3-K9 trimethylation is found in heterochromatin.³² We investigated the methylation status of H3-K9 on LS174T cells derived from the eye and from different organs since methylation of this lysine mark has been correlated with transcriptional repression^{33,34} in euchromatin. We found that the H3-K9 mark was preferentially dimethylated and trimethylated only by the LS174T-AC cells, suggesting that methylation at this particular lysine residue plays a critical role in silencing CXCR4 expression. Interestingly, the histone methyltransferase G9a has been shown to di- and trimethylate the H3-K9 mark to induce epigenetic suppression.³⁵ Moreover, Dong et al. and others have reported that G9a promotes DNA methylation by de novo methyltransferases.^{36,37} Additional research to determine whether H3-K9 methylation by G9a is responsible for silencing CXCR4 in our model will be performed to determine whether G9a is responsible for methylation at the H3-K9 mark in LS174T-AC cells and whether activated G9a is also associated with the upregulation of methyltransferase activity in these cells. Other epigenetic marks including H3-K4 and H3-K27 have also been shown to activate de novo methyltransferases and need to be considered in future studies.^{38,39} Future studies investigating the role of CXCR4 with other epigenetic marks such as H3-K4 may further our knowledge in the expression of CXCR4 gene and its possible regulation by histone interaction.

There is evidence that uveal melanoma phenotype is influenced by epigenetic mechanisms. Reduced p16^{INK4a} expression in uveal melanoma has been attributed to hypermethylation of the CpG islands in the p16^{INK4a} promoter region.⁴⁰ Methylation of the *ras*-association domain 1a (RASSF1a) and Ras and EF hand domain (RASEF) gene promoter regions has been associated with uveal melanoma tumor progression.^{41,42} Radosevich et al. proposed that absence of MHC class II expression by uveal melanoma cells is due to epigenetic silencing of the *CIITA* MHC class II regulator gene.⁴³ We previously reported that metastatic uveal melanoma cell lines derived from different metastatic liver nodules expressed the MAGE 1 tumor antigen.⁴⁴ Interestingly, one of the 4 cell lines tested (OMM 2.2), did not express MAGE 1. Treatment of OMM 2.2 with the demethylating agent 5-Aza 2-deoxycytidine restored MAGE 1 expression, suggesting that expression of MAGE tumor antigens in uveal melanoma is epigenetically regulated by the same epigenetic mechanisms that regulate MAGE expression in other tumors.⁴⁵ Together, these studies indicate that epigenetic mechanisms play a role in uveal melanoma tumor progression by regulating cell cycle progression and antigen presentation and expression.

We and others have demonstrated that primary uveal melanoma cell lines constitutively express CXCR4.^{15,46} Expression was at its highest levels in uveal melanoma cells that displayed epithelioid morphology,⁴⁶ which is a prognostic indicator for high malignancy. We also demonstrated that

CXCR4 expression was reduced in uveal melanoma cell lines injected into the anterior chamber of nude mice, but the expression was not as profound as what we observed by the injection of wild-type LS174T cells into the anterior chamber. It is possible that intrinsic differences between uveal melanoma and colon cancer cell morphology and differences in the local environment of the eye and colon could explain the differences in the levels of CXCR4 expression in anterior chamber-derived cell lines.

In summary, this study demonstrates that factors in the ocular environment induce epigenetic events that downregulate expression of CXCR4 in tumor cells. Identification and characterization of these factors may lead to their use in mitigating tumor cell migration to specific organs mediated by tumor cell expression of chemokine receptors, thereby reducing the incidence of metastasis.

Acknowledgments

The authors thank Wanhua Wang for her technical assistance.

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