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Regulation of Surfactant Protein B Gene Expression in Bone Marrow-Derived Cells

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ABSTRACT

While investigating the differentiation potential of bone marrow-derived cells, we previously demonstrated upregulated expression of the lung-related surfactant protein B (SP-B) gene in hematopoietic progenitor cells (HPCs) when they were cocultured with macerated lung tissue. During coculture, HPCs differentiated toward a dendritic-like myeloid cell phenotype (hematopoietic progenitor cell-derived dendritic-like cells [HPC-DCs]). However, immature dendritic cells (iDCs) cocultured under identical conditions did not express SP-B mRNA before or after coculture. We have now further examined the regulation of SP-B expression in HPC-DCs and iDCs. Of the transcription factors involved in SP-B gene expression, neither cell type expressed TTF-1, HNF3α, or HNF3β, but both cell types expressed Sp1 and Sp3. Sp1 binding to the SP-B promoter was investigated in these cells. Three novel Sp1 binding motifs were identified in the mouse SP-B promoter. Using chromatin immunoprecipitation, it was demonstrated that Sp1 was bound to all three sites in HPC-DCs after coculture with lung tissue, but not in iDCs. We hypothesized that although genes from multiple lineages may be active in HPCs, gene silencing events, such as methylation, may subsequently occur to suppress expression of these genes in more mature myeloid cells, such as iDCs. Treatment with the demethylating agent 5-azacytidine resulted in expression of the SP-B gene in iDCs. These data indicate that tissue-specific transcription factors are not required to express the lung-related gene SP-B in hematopoietic progenitor cells. Furthermore, silencing events, such as methylation, may occur to suppress lung-related gene expression as progenitor cells become committed toward more mature hematopoietic cell phenotypes. STEM CELLS *2009;27:662–669*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Reports that bone marrow (BM)-derived cells can engraft in vivo in lungs and differentiate into lung epithelial cells remain controversial (reviewed in [1]). Most studies have involved delivery of donor BM-derived cells to recipient mice that have some form of lung injury. Subsequent apparent coexpression of donor markers, such as the Y chromosome or green fluorescent protein, with lung epithelial cell markers, usually cytokeratins or surfactant proteins, is proposed to represent differentiation of BM-derived cells into lung epithelial cells. However, difficulties associated with several of these in vivo studies include verification that lung epithelial cells arose from BM-derived cells, identification of the specific type(s) of BM-derived cell that engrafts, and whether differentiation or fusion is occurring.

To address some of these issues, we previously reported an in vitro coculture system in which BM-derived cells are physically separated from mechanically macerated lung tissue obtained from otherwise healthy animals [2]. We hypothesized that if differentiation of BM-derived cells is occurring in injured lungs in vivo, it is likely that soluble lung-derived factors, such as those released from disrupted lung cells and extracellular matrices during maceration, are involved. We therefore examined the ability of hematopoietic progenitor cells (HPCs) to express lung-related genes in response to these soluble factors. When HPCs were cocultured with macerated lung tissue, upregulation of surfactant protein B (SP-B), cytokeratin 7, and cytokeratin 18 gene expression occurred, indicating that soluble lung-derived factors can upregulate lung-related gene expression in BM-derived cells in the absence of fusion [2]. The HPCs differentiated toward a dendritic cell-like population (hematopoietic progenitor cell-derived dendritic-like cells [HPC-DCs]) during coculture. However, when immature dendritic cells (iDCs) were first derived in vitro from cultured BM and subsequently placed in coculture with lung tissue, no expression of lung-related genes was detected either before or after culture. We concluded that lung-related gene expression was permissible only in more immature myeloid precursor cells under these coculture conditions.

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The long-standing paradigm of stem cell differentiation proposes that lineage-specific genes are inactive in stem cells and progenitor cells and are subsequently induced during tissue-specific differentiation. However, a new concept for the process of stem cell differentiation has been emerging in recent years. It has been suggested that stem cells in fact express a wide range of genes characteristic of the multiple lineage paths that these cells may be capable of adopting and that specific genes are silenced as cells progress along a particular differentiation pathway [3]. In support of this theory are reports describing expression of multilineage genes within progenitor cells. Isolated single bone marrow stromal cells, for example, simultaneously express transcripts characteristic of epithelial, endothelial, osteoblast, fibroblast, muscle, and neural/glial cells [4].

We were interested in whether a similar process occurs in bone marrow-derived HPCs and so examined mechanisms underlying regulation of expression of the lung-related SP-B gene in progenitor cells (HPC-DCs) and more mature cells (iDCs) in our lung coculture system. Despite their similar dendritic cell (DC)-like phenotypes following coculture, SP-B mRNA expression was detected only in HPCs following coculture with macerated lung tissue, and not in iDCs. We examined expression of transcription factors known to induce SP-B gene expression but found no difference in transcription factor profile that would explain SP-B gene expression in HPC-DCs and its absence from iDCs. We therefore hypothesized that although SP-B gene expression was permissible in HPCs, gene silencing events in more mature myeloid cells, such as iDCs, could prevent expression of SP-B in these cells. Gene silencing occurs when DNA is packaged around histones into nucleosomes. Modifications of histone proteins and DNA, including phosphorylation, methylation, and acetylation, alter DNA-histone interactions, thereby governing DNA-transcription factor interactions.

In the present study, we identified three previously undescribed putative Sp1 binding motifs in the mouse SP-B promoter and used chromatin immunoprecipitation to examine Sp1 binding to these motifs. We then used the demethylating agent 5-azacytidine to determine whether methylation was a potential mechanism of silencing SP-B gene expression in iDCs. Our data indicate that Sp1 activity is sufficient for expression of the SP-B gene in HPC-DCs and that methylation suppresses expression of SP-B in more mature iDCs.

Materials and Methods

Coculture of Hematopoietic Progenitor Cells

Bone marrow cells were isolated from 6-8-week-old female C3H-Hen mice (Harlan, Bicester, U.K., http://www.harlan.com). Care of animals was in accordance with institutional guidelines. Following cervical dislocation, whole bone marrow was obtained by flushing the femurs and tibias with an ice-cold solution of phosphate-buffered saline with 0.5% bovine serum albumin and 2 mM EDTA (Sigma-Aldrich, Dublin, Ireland, http://www.sigmaaldrich.com). Red blood cells were lysed using an RBC Lysis Solution (BioLegend, San Diego, http://www.biolegend.com). A cell population enriched for HPCs was obtained from the remaining cell suspension using a lineage depletion-negative selection kit according to the manufacturer's instructions (R&D Systems Inc., Abingdon, U.K., http://www.rndsystems.com). Mature lineage-positive cells were depleted magnetically using antibodies to CD5, CD11b, CD45R, Ly-6G, and Ter119. Cocultures were set up in a complete bone marrow medium consisting of RPMI 1640 supplemented with 13.5% (vol/vol) heat-inactivated fetal bovine serum (Gibco, Poole, U.K., http://www.invitrogen.com), 2 mM L-glutamine (Gibco), 5 ng/ml (wt/vol) gentamicin (Sigma-Aldrich), and 10% (vol/vol) conditioned media from Giant Cell Tumor (Sigma-Aldrich). Four grams (weight wet) of lung tissue was placed in 6 ml of complete bone marrow medium and minced to approximately 1-mm^3 cubes. Eight hundred microliters of medium only or tissue suspension was placed in each well, and the insert was added. A 330-μl cell suspension enriched for HPCs containing 5×10^5 cells per milliliter was added to the insert. Cocultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Culture of Immature Dendritic Cells

Whole bone marrow was obtained by flushing the femurs and tibias of 6-8-week-old female C3H-Hen mice with a warm solution of RPMI-1640 (Sigma-Aldrich) containing 1% (vol/vol) Penicillin-Streptomycin (Gibco). Dendritic cells were cultured as previously described with minor modifications (cells were harvested from the suspension at day 7) [5]. Briefly, bone marrow cells were cultured in complete medium supplemented with 150 μg/ml granulocyte macrophage-colony-stimulating factor (GM-CSF). After 3 days, suspension cells were discarded, and fresh medium was added to the adherent cells. After a further 4 days, iDCs were harvested as nonadherent cells from the medium. Cells were cocultured as described above but in six-well inserts in complete medium supplemented with 150 μg/ml GM-CSF. One milliliter of cell suspension was seeded into six-well tissue culture plate inserts. Inserts were placed into six-well plates containing 2 ml of control medium or lung tissue suspension per well.

Characterization of Cells by Flow Cytometry

The following antibodies were used to characterize cells: CD11b-phycoerythrin (PE), CD11c-fluorescein isothiocyanate (FITC), F4/80-FITC, and major histocompatibility factor (MHC)-II-PE (eBioscience Inc., San Diego, http://www.ebioscience.com). Appropriate isotype control antibodies were used (eBioscience). Cells (1×10^5) were incubated with 0.5 μl of antibody for 10 minutes at 4°C. Cells were washed, and flow cytometry was performed using a FACSCalibur instrument (Becton, Dickinson and Company, Cowley, U.K., http://www.bd.com) and analyzed using CellQuest software (Becton Dickinson).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from cells using TriZol (Invitrogen, Paisley, U.K., http://www.invitrogen.com) according to the manufacturer's instructions. Samples were treated with DNase I, Amp Grade (Invitrogen), for 25 minutes, and cDNA was synthesized using Moloney murine leukemia virus Reverse Transcriptase (Invitrogen). Polymerase chain reaction (PCR) primers were designed using Primer 3′ web software (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, http://www.wi.mit.edu). PCR consisted of 35 cycles: 94°C for 45 seconds (denaturation), 55°C–58°C for 45 seconds (annealing), and 72°C for 1 minute (extension) using a PTC-100 programmable thermal cycler (MJ Research, Inc., Quebec, Canada, http://www.mj-research.com). Murine airway epithelial cells, isolated as previously described [6], were used as a positive control. PCR primers are listed in Table 1.

Western Blotting

Approximately 4×10^6 cells were lysed in 30 μl of RIPA buffer (150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris) supplemented with 1× Complete Mini protease inhibitor cocktail (Roche, Dublin, Ireland, http://www.roche-applied-science.com). Fifteen micrograms of protein was electrophoresed on 12% SDS-polyacrylamide gel electrophoresis gels and blotted onto nitrocellulose membrane. Blots were probed with glyceraldehyde-3-phosphate dehydrogenase (Millipore, Carrigtwohill, Ireland, http://www.millipore.com), Sp1 (Active Motif, Rixenhart, Belgium), and Sp3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) primary antibodies followed by the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by chemiluminescence.

Table 1. List of primers

Gene	Primer	T_A (°C)	Product (bp)
RT-PCR			
GAPDH	F: 5′-CTGCACCACCAACTGCTTAG-3′	55	487
	R: 5′-CCAGGAAATGAGCTTGACAAA-3′		
SP-B	F: 5′-CTACCTGCCCCTGGTTAT-3′	58	549
	R: 5′-GACTTGCAGAAATGGCAC-3′		
TTF-1	F: 5′-GTGCCGGTCCTAGTCAAAGA-3′	58	273
	R: 5′-CAGATGGGATAGGCTGGAGA-3′		
HNF3α	F: 5′-CGCTTCGGAGTTGAAGTCTC-3′	58	372
	R: 5′-AGCACGGGTCTGGAATACAC-3′		
HNF3β	F: 5′-CTCTTCCGTGAGCAACATGA-3′	58	392
	R: 5′-TGTAGCTGCGTCGGTATGTC-3′		
Sp1	F: 5′-TTCTCAGACTCGAAGCAGCA-3′	58	346
	R: 5′-CACAACATACTGCCCACCAG-3′		
Sp3	F: 5′-CTTTGACGCCTGTTCAGACA-3′	55	374
	R: 5′-ACCTCTCCCACCACCTTCTT-3′		
ChIP			
SP-B1	F: 5′-CCACAGGGGACACAGAAATC-3′	57	387
	R: 5′-CGATGTCGGTTCCTAGTCCT-3′		
SP-B2	F: 5′-TGCTGGAGGACTTTCTAGGG-3′	57	181
	R: 5′-TGAGGCTATGTCCACACCTG-3′		
SP-B3	F: 5′-CAGTGCTCACCTGTATGTGG-3′	58	208
	R: 5′-CCCAAGTGCTGGGATTAAAA-3′		
CD11b1	F: 5′-TGTTTTTACCCCTCCCTCCT-3′	58	189
	R: 5′-CAATCTGAGGACCCCCTTCT-3′		
CD11b2	F:-5′-AGCCTGATCCGAAACACTGA-3′	56	260
	R: 5′-AGGAGGGAGGGGTAAAAACA-3′		

Abbreviations: bp, base pairs; ChIP, chromatin immunoprecipitation; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; R, reverse; RT-PCR, reverse transcription-polymerase chain reaction; SP-B, surfactant protein B; T_A, annealing temperature.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was carried out using approximately 6×10^6 cells using the ChipIT-Express kit according to the manufacturer's instructions (Active Motif, Rixensart, Belgium, http://www.activemotif.com). PCR consisted of 36 cycles: 94°C for 30 seconds, 56°C–58°C for 20 seconds, and 72°C for 20 seconds. Chromatin immunoprecipitation (ChIP) primers are listed in Table 1.

Culture with 5-Azacytidine

At day 3 of the 7-day culture to derive iDCs, 5-azacytidine (5-aza) (Sigma-Aldrich) was added to a final concentration of 40 μM and was replaced with fresh medium 24 hours later. Cell numbers and viability were monitored by ethidium bromide/acridine orange staining (Sigma-Aldrich). On day 7, iDCs were harvested and placed in coculture with lung tissue as described above.

Results

Expression of SP-B and SP-B-Related Transcription Factors in HPC-DCs and iDCs After Coculture with Lung Tissue

We previously characterized HPCs to be $lin^{[-]}$ c-Kit^+ $CD34^{lo}$ $Sca1^{lo}$ [2]. When these HPCs are exposed to soluble signals from macerated lung tissue, they differentiate toward a DC-like phenotype, HPC-DC, that expresses increased levels of SP-B mRNA compared with either freshly isolated HPCs or HPCs cultured with control medium. It is possible that these lung-derived soluble signals are capable of upregulating SP-B gene expression only in HPCs that subsequently differentiate into DC-like cells that then retain these higher levels of SP-B mRNA. Alternatively, upregulation of SP-B gene expression may occur after HPCs begin to differentiate toward DCs. To explore these possibilities, we generated iDCs in vitro and then examined their ability to upregulate SP-B gene expression when cocultured with macerated lung tissue. iDCs were first derived by culturing bone marrow cells in the presence of GM-CSF for 7 days and subsequently cocultured with lung tissue for 3 days. HPC-DCs were derived by culturing HPCs with lung tissue for 3 days. The resulting iDC and HPC-DC cell phenotypes were compared.

The HPC-DC population was phenotypically similar to iDCs after coculture (Fig. 1). Both populations were $CD11b^{hi}$, although iDCs expressed more CD11c and F4/80. The iDC population was more uniform in its expression of CD11c, although the HPC-DCs may have differentiated into a more mixed myeloid population. HPC-DCs cocultured with lung tissue expressed SP-B mRNA, whereas no SP-B mRNA was detected in cocultured iDCs (Fig. 2A).

TTF-1, HNF3α, and HNF3β are lung-related transcription factors that regulate expression of several lung-related genes and are the primary factors that regulate SP-B gene expression [7–9]. Sp1 and Sp3 are ubiquitous transcription factors that regulate a wide range of genes in most tissue types and are also involved in transcriptional regulation of SP-B [9]. To examine whether differences in the profiles of SP-B-related transcription factors could explain the absence of SP-B transcripts in iDCs, expression of these transcription factors was determined in HPC-DCs and iDCs after 3 days of coculture with lung tissue. MLE-12 mouse lung cells express SP-B and were used for comparison.

Strong expression of all transcription factor genes was detected in MLE-12 cells (Fig. 2A). However, neither HPC-DCs nor iDCs expressed TTF-1, HNF3α, or HNF3β genes after coculture. Sp1 and Sp3 mRNA and protein were detected in both HPC-DCs and iDCs (Fig. 2A, 2B). Levels of Sp1 protein were similar in HPC-DCs, iDCs, and MLE-12 cells. Sp3 protein levels were higher in iDCs compared with HPC-DCs.

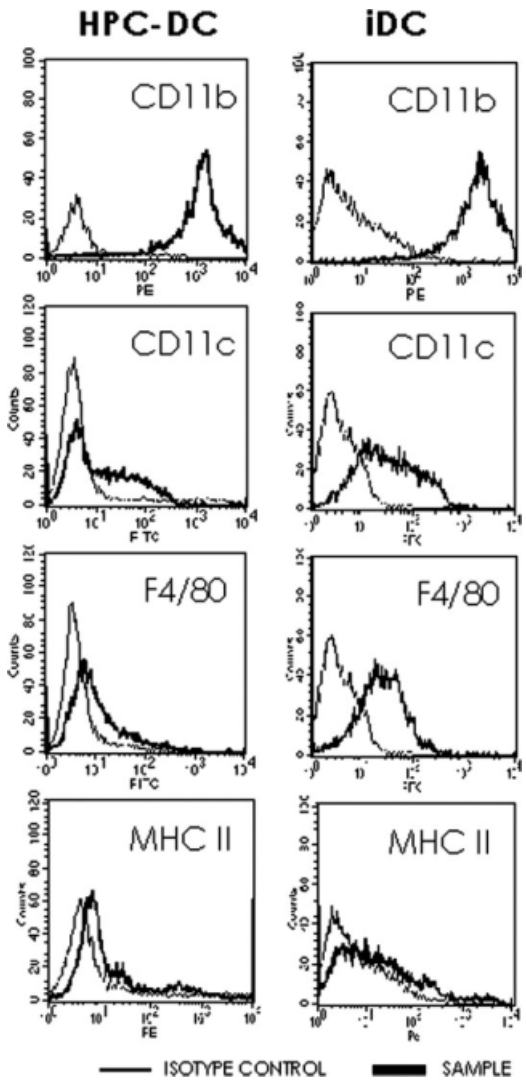

Figure 1. Characterization of HPC-DCs and iDCs after coculture with macerated lung tissue. We have previously shown that HPCs differentiate into myeloid dendritic-like cells (HPC-DCs) when cocultured with lung tissue for 3 days [2]. We now show that iDCs have a similar phenotype after 3 days in coculture with lung tissue. Graphs are representative of three separate experiments. Abbreviations: FITC, fluorescein isothiocyanate; HPC-DC, hematopoietic progenitor cell-derived dendritic-like cell; iDC, immature dendritic cell.

Analysis of Sp1 Binding Sites in the Mouse SP-B Promoter

Because neither HPC-DCs nor iDCs express the lung-related transcription factors normally required for SP-B gene expression, we hypothesized that random Sp1 and Sp3 activity may be responsible for inducing SP-B expression in HPCs. Because Sp3 expression was relatively low in HPC-DCs despite upregulated SP-B expression, we decided to examine whether differences in Sp1 activity between HPC-DCs and iDCs could explain the presence of SP-B transcripts in the former and their absence from the latter.

Although Sp1 binding sites have previously been identified in the proximal region of both the human and rabbit SP-B promoters [7, 9], no Sp1 sites have been described in the mouse SP-B promoter to date. The minimal Sp1 binding motif is 5′-GCGGG-3′ [10]. An alignment of the proximal regions of the human, rabbit, and mouse SP-B promoter demonstrates that the Sp1 sites identified in the rabbit promoter are not present in the mouse promoter (Fig. 3A). We therefore analyzed the SP-B promoter for putative Sp1 binding sites. Deletion analysis has previously shown that the region downstream of −842 base pairs (bp) is important in the control of transcription of the mouse SP-B gene [11]. Furthermore, distally bound Sp1 located up to 1.8 kilobases away can interact with proximally bound Sp1 by looping out the intervening DNA and acting in a synergistic manner [12, 13]. Therefore, the region between +1 and −2,000 bp was analyzed for Sp1 binding motifs. Three putative Sp1 sites were identified in the mouse SP-B promoter at −336, −733, and −1,243 bp (Fig. 3B).

Chromatin Immunoprecipitation

ChIP was carried out to confirm these novel Sp1 binding sites in the mouse SP-B gene and to examine potential differential Sp1 activity at these sites in HPC-DCs compared with iDCs after coculture with lung tissue. MLE-12 cells were used as a positive technical control. PCR primers sets were designed to span each of the three putative Sp1 sites, designated primer sets 1, 2, and 3 (Fig. 4A). An additional two previously unreported Sp1 binding motifs were identified in the CD11b promoter, and primers sets were designed to span these (Fig. 4B). Sp1 binding to the CD11b promoter was used as an internal control as both HPC-DCs and iDCs express high levels of CD11b, whereas MLE-12 cells do not. IgG control antibodies were used to control for nonspecific DNA amplification, which was subtracted from that detected with the anti-Sp1 antibody.

DNA surrounding the three putative Sp1 sites in the SP-B promoter was immunoprecipitated using an anti-Sp1 antibody. Significantly higher levels of DNA were detected for all three sites in cocultured HPC-DCs compared with iDCs, indicating a greater degree of occupancy of these Sp1 sites in HPC-DCs (Fig. 4C). DNA surrounding the putative Sp1 sites in the CD11b promoter was also detected in both HPC-DCs and iDCs, with significantly less DNA detected from the MLE-12 cells as predicted.

Demethylation of the SP-B Promoter in iDCs

Having confirmed that the three putative Sp1 sites in the SP-B promoter were capable of binding Sp1 and that a higher level of occupancy occurred in HPC-DCs cocultured with macerated lung tissue compared with cocultured iDCs, we hypothesized that methylation of the SP-B promoter may render these Sp1 sites inaccessible in iDCs. Therefore, we examined the effects of the demethylating agent 5-aza on SP-B gene expression in iDCs.

5-Aza was added on day 3 of the 7-day culture period in which bone marrow cells were cultured with GM-CSF to generate iDCs. Treatment with 5-aza resulted in a 30% decrease in the yield of iDCs at day 7 compared with cultures that did not receive 5-aza. However, treated cultures remained 98% viable and were phenotypically similar to untreated iDCs, as determined by their expression of CD11b, CD11c, F4/80, and MHC II (data not shown). 5-Aza-treated and untreated iDCs were subsequently cocultured with lung tissue or in medium-only control for 3 days. SP-B gene expression was determined by reverse transcription-PCR.

Induction of SP-B gene expression was evident in 5-aza-treated iDCs, both in medium-only controls and in cocultures with lung tissue (Fig. 5). Nested PCR was carried out to further amplify the SP-B product. It was possible that 5-aza treatment induced expression of transcription factors, which in turn resulted in SP-B expression rather than direct demethylation of the SP-B promoter itself. Expression of TTF-1,

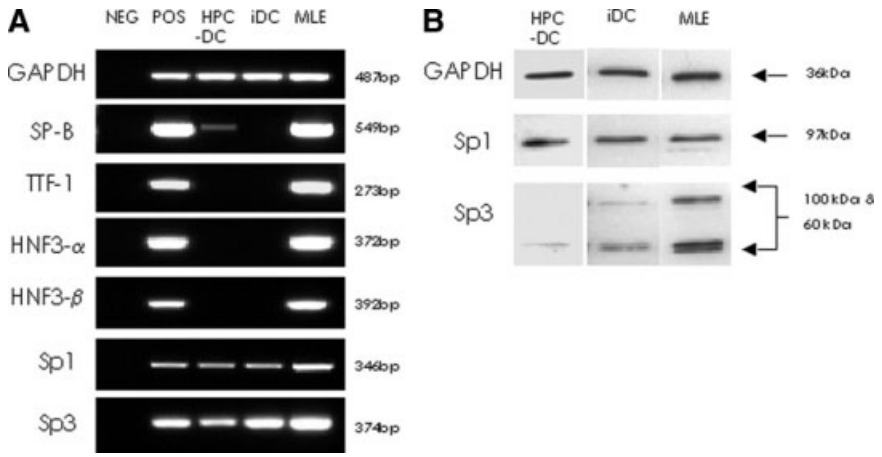

Figure 2. Expression of surfactant protein B (SP-B) and SP-B-related transcription factors in HPC-DCs and iDCs after coculture with macerated lung tissue. **(A):** Reverse transcription-polymerase chain reaction HPC-DCs expressed low levels of SP-B mRNA after coculture with lung tissue. In contrast, iDCs did not express the SP-B gene after coculture. Neither cell type expressed the lung-related transcription factors TTF-1, HNF3α, or HNF3β genes normally required for transcription of the SP-B gene. POS and MLE both expressed high levels of the SP-B gene and the lung-related transcription factors. Image is representative of three separate experiments. **(B):** Western blot. HPC-DCs cocultured with lung tissue expressed low levels of Sp3 protein compared with iDCs or MLE. Cells expressed comparable levels of Sp1 protein. Image is representative of two separate experiments. Abbreviations: bp, base pairs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPC-DC, hematopoietic progenitor cell-derived dendritic-like cell; iDC, immature dendritic cell; MLE, MLE-12 lung cell line; NEG, control, omitting cDNA; POS, normal primary lung epithelial cells.

HNF3α, HNF3β, Sp1, and Sp3 genes was therefore also examined. Neither untreated nor 5-aza-treated iDCs expressed TTF-1, HNF3α, or HNF3β after coculture with lung tissue. Untreated and 5-aza-treated iDCs expressed similar levels of Sp1 and Sp3 genes in medium only and in cocultures with lung tissue.

Levels of SP-B mRNA did not appear increased in iDCs cocultured with lung tissue compared with medium-only controls. Therefore, we deduced that demethylation of the SP-B promoter was sufficient to allow transcription of SP-B in the absence of signals from the lung tissue. We therefore examined SP-B expression in iDCs at day 7 after treatment with 5-aza, without further coculture. Treatment with 5-aza was sufficient to allow weak expression of SP-B, as shown by nested PCR (Fig. 6A). Chromatin immunoprecipitation was carried out to investigate Sp1 binding to the SP-B promoter regions as before. Although to a lower degree than in HPCs, there was increased occupancy of Sp1 at the SP-B promoter regions in 5-aza-treated iDCs compared with untreated iDCs (Fig. 6B).

We therefore conclude that SP-B expression in 5-aza-treated iDCs occurred, at least in part, as a result of demethylation of the SP-B promoter and subsequent induction by Sp1. This occurred in the absence of lung-specific signals.

Discussion

Surfactant protein expression is widely used as a lung cell marker in studies of bone marrow-derived cell differentiation into lung epithelial cells in vivo. However, several issues remain to be clarified in these studies, including establishing the identity of the bone marrow cell type that has ultimately engrafted into the lungs and definitive confirmation that lung markers are expressed in these engrafted cells. We previously examined SP-B expression in bone marrow-derived HPCs following exposure to soluble signals from macerated lung tissue in coculture. Over the course of the coculture, HPCs differentiated toward a dendritic-like myeloid phenotype, HPC-DC, in which SP-B mRNA levels were increased. However, when iDCs were cocultured with lung tissue, SP-B mRNA was not detected before or after coculture. In that study we assessed the possibility of crossover of cellular material or DNA-containing vesicles in the coculture. Aliotta et al. [14] demonstrated that DNA-containing vesicles can be released from lung tissue and phagocytosed by myeloid cells. We found no evidence of this in our coculture system [2].

In the present study, we hypothesized that upregulation of SP-B occurs in HPC-DCs when cocultured with macerated lung tissue because they receive appropriate signals while they are immature progenitor cells during the culture period, before they differentiate toward a DC-like phenotype. In contrast, SP-B expression is not permissible in more mature iDCs in response to the same signals because of differences in either transcriptional or epigenetic regulation of the SP-B gene in these cells, or both. We therefore determined the SP-B transcription factor profile in both cell types and examined methylation of the SP-B promoter.

We discovered that neither HPC-DCs nor iDCs express the lung-related transcription factors TTF-1, HNF3α, and HNF3β, which would be expected to induce transcription of the SP-B gene in lung cells (Fig. 2A). Sp1 and Sp3 proteins were present in both HPC-DCs and iDCs (Fig. 2B). However, although Sp1 protein was expressed at a similar level in both HPC-DCs and iDCs, Sp3 protein levels were very low in HPC-DCs. Of these two proteins, it was therefore more likely that Sp1 was potentially involved in regulating transcription of SP-B in HPC-DCs, and this transcription factor was examined further. No Sp1 sites have been previously described in the mouse SP-B promoter. However, the importance of Sp1 for expression of the rabbit SP-B gene has been demonstrated by promoter deletion analysis, whereby deletion of a single Sp1 site decreased SP-B gene expression by 80% [9]. It has also been shown that coexpression of Sp1 alone with the proximal promoter region of the rabbit SP-B gene increased CAT reporter expression significantly more than expression of TTF-1, HNF3α, or HNF3β [15]. Expression of Sp1 alone resulted in CAT reporter activity similar to that of combined coexpression of Sp1, TTF-1, HNF3α, and HNF3β. Our

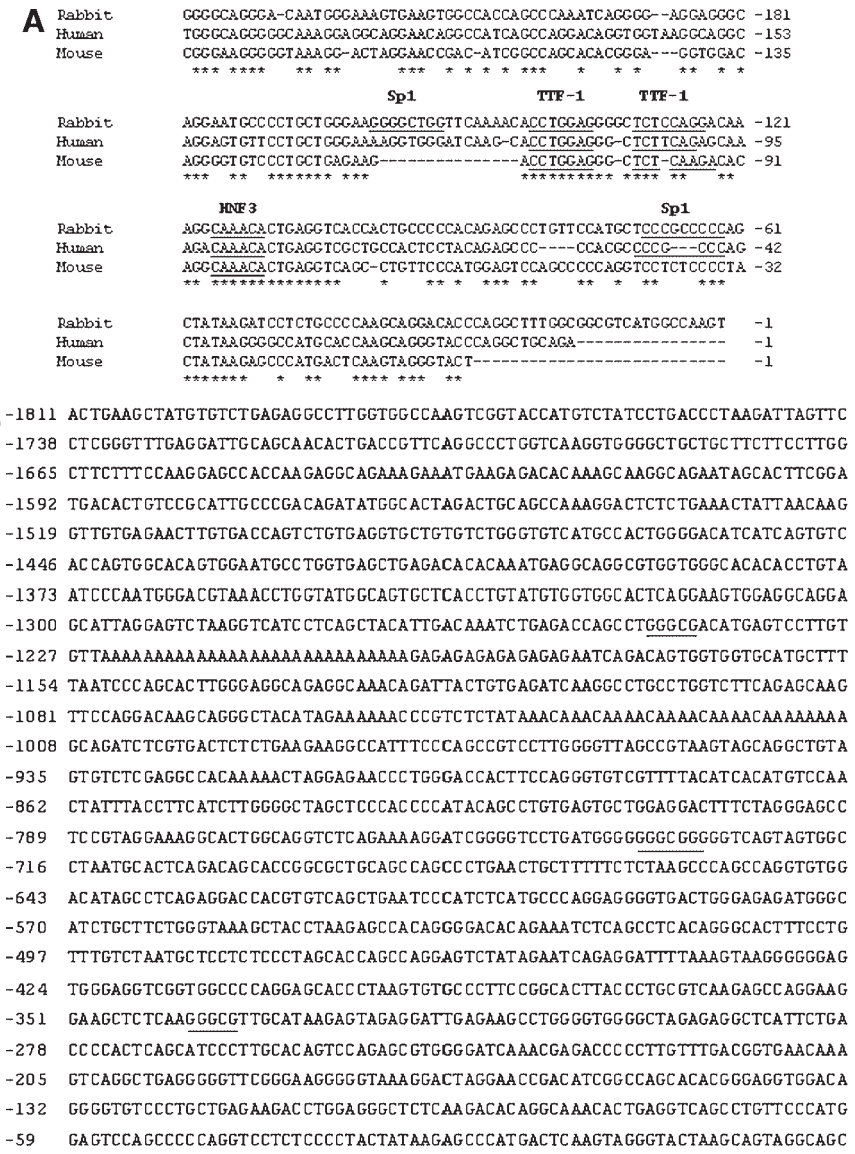

Figure 3. Analysis of Sp1 binding motifs in the mouse surfactant protein B promoter. **(A):** The TTF-1 binding sites and HNF3 binding site that make up the proximal enhancer region of the SP-B promoter are highly conserved in rabbit, human, and mouse. The Sp1 binding sites in the rabbit promoter are not present in the mouse promoter. **(B):** Three putative Sp1 sites were identified, at −336, −733, and −1,243 base pairs according to the minimal binding motif 5′-GCGGG-3′ [10].

analysis revealed three novel putative Sp1 binding motifs in the mouse SP-B promoter at −336, −733, and −1,243 bp (Fig. 3B). Chromatin immunoprecipitation confirmed that these are Sp1 binding sites and that Sp1 interacts with each of these sites in HPC-DCs but not in iDCs. Ideally, epithelial cells derived from the macerated lung tissue would have been used as a positive control in the ChIP and demethylation experiments in this study. However, it would be extremely technically challenging to obtain sufficient numbers of epithelial cells from macerated lung tissue for this control. Therefore, the MLE-12 murine lung cell line was used as a positive technical control to demonstrate binding of Sp1 to the SP-B promoter. Sp1 interaction with the CD11b promoter was examined as an internal control, and Sp1 was shown to be bound to regions of the CD11b promoter containing Sp1 motifs in both HPC-DCs and iDCs after coculture (Fig. 4C). As expected, there was little or no binding of Sp1 to the CD11b promoter detected in MLE-12 cells, which do not express this gene. Thus, Sp1 is bound to the SP-B promoter in HPC-DCs exposed to signals from macerated lung tissue and is likely to be upregulating of SP-B gene expression in these cells. In contrast, although iDCs contain Sp1 protein, Sp1 does not interact with the SP-B promoter in these cells in response to signals from lung tissue.

We hypothesized that gene silencing events in more mature committed iDCs may prevent the Sp1 transcription factor from accessing the Sp1 sites in the promoter. Methylation of cytosine residues within promoter regions can obstruct transcription factor binding and recruit histone deacetylases. Several studies have suggested that methylation of adjacent cytosines or upstream CpG dinucleotides can affect Sp1 binding [16–18].

To determine whether methylation was preventing SP-B transcription in more mature myeloid cells, iDCs were derived from bone marrow cells in the presence of the demethylating agent 5-azacytidine. Treated and untreated iDCs were subsequently cocultured with lung tissue or in medium-only controls. SP-B gene expression was detected in 5-aza-treated iDCs both in medium-only controls and in coculture with lung tissue (Fig. 5). Nested PCR confirmed the expression of SP-B in 5-aza-treated iDCs. Even with these more sensitive conditions, no SP-B mRNA was detected in untreated iDCs. 5-Aza treatment did not induce expression of TTF-1, HNF3α, or HNF3β, and untreated and 5-aza-treated iDCs expressed

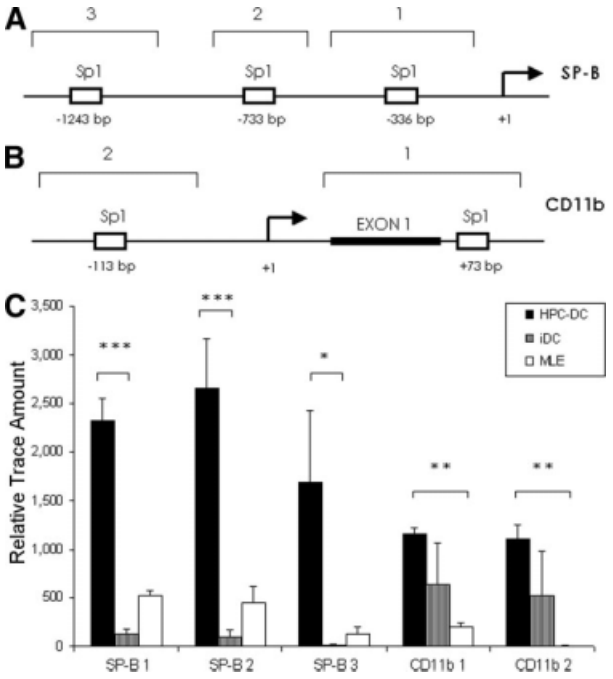

Figure 4. Chromatin immunoprecipitation. **(A):** Three primer sets, denoted by 1, 2, and 3, were designed to span the three Sp1 binding motifs identified in the msSP-B promoter (Table 1). **(B):** Two primer sets, denoted by 1 and 2, were designed to span the two Sp1 binding motifs identified in the CD11b promoter (Table 1). **(C):** After 3 days of coculture with macerated lung tissue, levels of Sp1 binding to the DNA regions spanned by primers denoted by SP-B1, SP-B2, and SP-B3 were higher in HPC-DCs compared with iDCs. Binding of Sp1 to Sp1 motifs in the CD11b promoter was examined as an internal control. Levels of Sp1 binding to DNA spanned by primers denoted by CD11b1 and CD11b2 were higher in both HPCs and iDCs after 3 days of coculture compared with MLE-12 cells. Graph represents the mean from three separate experiments with the SD. *, $p < .05$; **, $p < .01$; ***, $p < .001$; using a one-way analysis of variance and Tukey's post test. Abbreviations: bp, base pairs; HPC-DC, hematopoietic progenitor cell-derived dendritic-like cell; iDC, immature dendritic cell; SP-B, surfactant protein B.

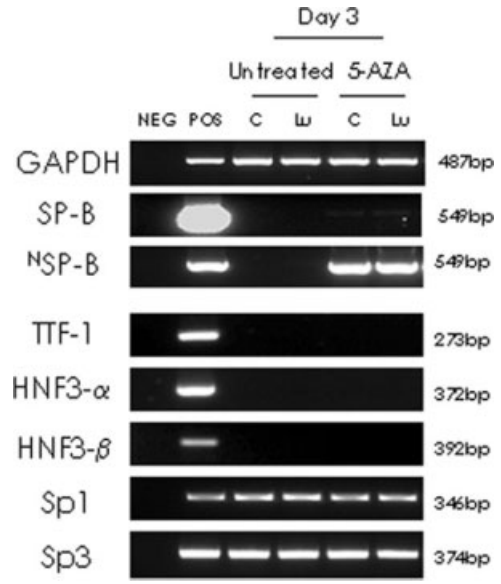

Figure 5. Gene expression in immature dendritic cells (iDCs) after treatment with 5-azacytidine. 5-Azacytidine induced weak expression of SP-B both in medium-only controls and in cocultures with macerated lung tissue. ^{N}SP-B confirmed expression of SP-B in 5-azacytidine-treated iDCs after coculture for 3 days in medium only and in Lu. No difference in expression of transcription factors involved in SP-B expression was evident in 5-azacytidine-treated versus untreated iDCs. Image is representative of two separate experiments. Abbreviations: 5-AZA, treated with 5-azacytidine; bp, base pairs; C, medium-only control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Lu, coculture with lung tissue; NEG, control, omitting cDNA; ^{N}SP-B, nested polymerase chain reaction; POS, normal primary lung epithelial cells; SP-B, surfactant protein B.

similar levels of Sp1 and Sp3 genes in medium only and in cocultures with lung tissue. Therefore, the induction of SP-B expression was not due to induced expression of transcription factors by the demethylating agent.

5-Aza-treated iDCs expressed a similar level of SP-B whether cultured in medium only or with macerated lung tissue. It appeared that the demethylating agent was sufficient to allow SP-B gene expression in the absence of signals from the lung tissue. Therefore, SP-B expression was examined in 5-aza-treated iDCs at day 7 before further coculture. Weak SP-B expression was detected (Fig. 6A). Chromatin immunoprecipitation demonstrated increased binding of Sp1 to the SP-B promoter region in iDCs treated with 5-aza compared with untreated cells (Fig. 6B). This suggests that demethylation of the SP-B promoter region is sufficient to allow SP-B expression in the absence of any lung-specific signals.

Coculture with macerated lung tissue may have resulted in demethylation of the SP-B promoter in HPCs, allowing Sp1 and/or Sp3 to upregulate expression of these genes. It is possible that the same coculture conditions were not sufficient to induce demethylation of the promoter in iDCs, whereas the more severe treatment with 5-azacytidine resulted in demethylation and subsequent Sp1/Sp3-mediated transcription.

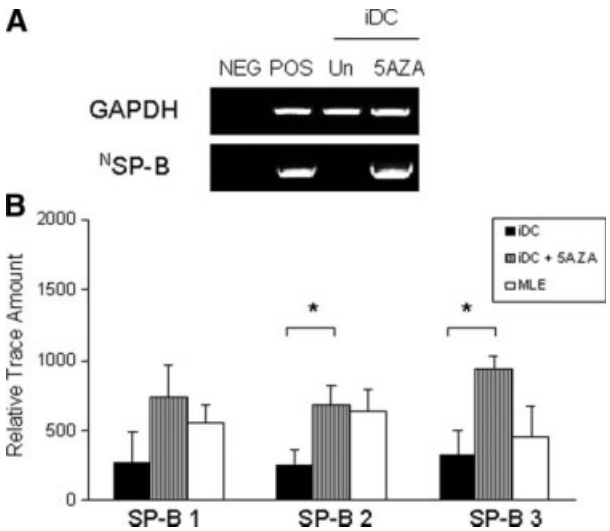

Figure 6. Sp1 binding to the SP-B promoter in iDCs after treatment with 5-azacytidine. **(A):** Treatment with 5-azacytidine resulted in weak SP-B gene expression in iDCs in the absence of lung-specific signals. Image is representative of three separate experiments. **(B):** Chromatin immunoprecipitation demonstrated increased levels of Sp1 binding to the SP-B promoter regions in iDCs after treatment with 5-azacytidine compared with Un. Graph represents the mean from three separate experiments with the SD. *, $p < .05$ using a one-way analysis of variance and Tukey's post test. Abbreviations: 5AZA, treated with 5-azacytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iDC, immature dendritic cell; NEG, control, omitting cDNA; ^{N}SP-B, nested polymerase chain reaction; POS, normal primary lung epithelial cells; SP-B, surfactant protein B; Un, untreated immature dendritic cell.

SUMMARY

Our study demonstrates that HPCs are capable of expressing the SP-B gene in the absence of lung-related transcription factors, such as TTF-1, HNF3α, or HNF3β. Our data indicate that demethylation of the SP-B promoter leads to increased Sp1 activity, which results in SP-B gene expression. Thus, SP-B gene expression can be detected in these HPCs in our coculture model system in the absence of lung-related differentiation. This raises doubt as to the utility of SP-B gene expression as a marker of lung-specific differentiation in HPCs in vivo. However, our data also demonstrate that bone marrow-derived cells can be reprogrammed in vitro to express genes that are important for lung homeostasis. It is possible that demethylation of promoter regions such as these Sp1 sites are an early step in this reprogramming. Whether this occurs in vivo remains to be determined.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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