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The SLE-associated Pbx1-d isoform acts as a dominant-negative transcriptional regulator

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Abstract

Pbx1 is a transcription factor involved in multiple cellular processes, including the maintenance of self-renewal of hematopoietic progenitors. We have shown that the CD4⁺ T-cell expression of a novel splice isoform of *Pbx1*, *Pbx1-d*, is associated with lupus susceptibility in the NZM2410 mouse and in lupus patients. The function of *Pbx1* in T cells is unknown, but the splicing out of the DNA-binding domain in *Pbx1-d* predicts a dominant-negative function. In support of this hypothesis, we have shown that *Pbx1-d* transduction accelerates differentiation of MC3T3-E1 osteoblast pregenerators and mimics the effect of short hairpin RNA silencing of *Pbx1*. Conversely, *Pbx1-d* transduction reduced the expression of *Sox3*, a gene strongly transactivated by Pbx1, and Pbx1-d did not bind the *Sox3* promoter. These results constitute a first step towards the understanding on how *Pbx1-d* contributes to systemic autoimmunity in the NZM2410 mouse model as well as in lupus patients.

Keywords

Lupus; T cells; Pbx1

INTRODUCTION

Sle1a1 is a lupus susceptibility locus that results in the production of activated anti-chromatin-specific CD4⁺ T cells with enhanced effector functions in the NZM2410 strain.^{1–3} *Sle1a1* expression also results in a reduced frequency of Foxp3⁺ regulatory CD4⁺ T cells,^{1,4} and *in vitro* experiments suggested that it is at least partially due to a defective response to *all trans* retinoic acid during TGFβ-mediated Foxp3 induction.^{3,5} The only gene located in the *Sle1a1* congenic interval is the pre-B-cell leukemia homeobox 1 (*Pbx1*) gene, a member of the three-amino-acid loop extension family of homeodomain-containing transcription factors that modulates the DNA-binding function of Hox proteins,⁶ and interacts with Meis and Prep1, two other three-amino-acid loop extension proteins that regulate chromatin remodeling and coactivator access.⁷ Pbx1 has a central role during development and organogenesis by integrating multiple signals through its interaction with

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

numerous partners.⁸ Therefore, *Pbx1*-deficient mice display an embryonic lethal phenotype.^{9,10} In the immune system, *Pbx1* preserves self-renewal of hematopoietic stem cells and blocks lineage-specific differentiation.¹¹ On the other hand, *Pbx1*-deficient embryonic stem cells fail to generate common lymphoid progenitors, resulting in the absence of B and NK cells, as well as an impaired T-cell development.¹² These seemingly opposite effects reflect the ability of Pbx–Meis complexes to contribute to either gene activation or repression by interacting with both Hox and non-Hox factors.⁸

CD4⁺ T-cell phenotypes associated with *Sle1a1* expression are T-cell intrinsic.³ The function of Pbx1 in T cells is unknown, although Pbx1 mutants that prevent Prep1/Pbx1 DNA binding have been reported to alter T-cell thymic development.¹³ Seven protein-coding *Pbx1* splice isoforms have been described, only two of which, *Pbx1-a* (*Pbx1-002*) and *Pbx1-b* (*Pbx1-001*), have known functions.^{14,15} *Pbx1-a* and *Pbx1-b* show differential temporal and cell-specific expression patterns, as well as distinct and sometimes opposite functions.⁸ *Pbx1-a* possesses a C-terminus domain that recruits corepressor proteins SMRT and NcoR.¹⁶ It has been hypothesized that *Pbx1-b* lacking exon 7 that is part of the HOX-binding domain is less transcriptionally active than *Pbx1-a*.¹⁴

We have shown that the major splice isoform expressed in CD4⁺ T cells is *Pbx1-b*, which lacks exon 7 and 9 that are present in *Pbx1-a*.³ In addition, NZM2410 CD4⁺ T cells express a novel splice isoform, *Pbx1-d* (*Pbx1-005*), which lacks exons 6 and 7 that encode the DNA and HOX-binding domains, respectively.³ Pbx1 amino-acid sequence is identical between human and mouse, and we found that *PBX1-d* is expressed at a significantly higher frequency in the CD4⁺ T cells of lupus patients as compared with healthy controls.³ *Pbx1-d* is the only known isoform lacking the DNA-binding domain, but it can compete for cofactors with either *Pbx1-a*, with which it shares terminal exon 9, or with *Pbx1-b*, with both Pbx1-b and Pbx1-d lacking exon 7.³ This suggested that the contribution of *Pbx1-d* to lupus susceptibility was through its function as a dominant negative.

To test this hypothesis, we used two *in vitro* models in which the role of *Pbx1* has been well characterized. *Pbx1-b* expression prevents osteoblast differentiation in MC3T3-E1 osteoblast progenitors by nucleating the formation of a transcriptional repression complex on the promoter of osteoblast-specific genes.¹⁷ Chromatin-bound Pbx1-b complexes recruit histone deacetylases that maintain chromatin inactivation on the promoters of osteoblast-specific genes. Loss of Pbx1-b binding to these promoters allows the recruitment of histone acetylases, as well as the decreased H3K9 methylation, reflecting transcriptional activation and osteoblast differentiation. We have used this model to address *Pbx1-d* function in the context of transcriptional repression. Pbx1 also contributes gene activation, with myogenesis being the best characterized model.⁸ *Sox3* (SRY-box 3) is a transcription factor that regulates neuronal differentiation.¹⁸ Pbx1 has been shown to be a critical factor for *Sox3* basal and *all trans* retinoic acid-induced expression.¹⁹ We have used the *Sox3* promoter to address *Pbx1-d* function in the context of transcriptional activation. With both models, we found that *Pbx1-d* expression was equivalent to knocking down Pbx1 expression, validating the hypothesis that it functions as a dominant negative. Furthermore, we showed that the Pbx1-d increased the expression of the CD44 activation marker in Jurkat T cells, and the same result was obtained by knocking down Pbx1 in this cell line. This finding constitutes a first step to determine *Pbx1-d* function and its contribution to autoimmunity.

RESULTS AND DISCUSSION

Pbx1-d accelerates osteoblast differentiation

To test the hypothesis that *Pbx1-d* acts as a dominant negative of *Pbx1* transcriptional repression, we compared the effect of *Pbx1-d* expression with that of *Pbx1* short hairpin

RNA (shRNA), which has been shown to accelerate MC3T3-E1 cell differentiation into osteoblasts.¹⁷ MC3T3-E1 cells express *Pbx1-b*, and transduction with *Pbx1-d* lentivirus (LV) resulted in the expression of high levels of *Pbx1-d* in addition to the endogenous *Pbx1-b* (Figure 1a). Transduction of these cells with *Pbx1-b* LV resulted in over a twofold increase of *Pbx1-d* expression, whereas *Pbx1* shRNA nearly eliminated *Pbx1-b* expression (Figure 1a). Osteoblast differentiation was first determined by alkaline phosphatase activity (Figures 1b and c). *Pbx1-d* expression significantly increased alkaline phosphatase activity as compared with empty vector (EV) transduction, and this was comparable to shRNA *Pbx1* knockdown. Interestingly, both *Pbx1-d* and *Pbx1* shRNA also increased alkaline phosphatase activity in MC3T3-E1 cells in the absence of differentiation induction. These results were confirmed by analysis of *Alp* message expression as well as that of *Bsp* and *Ocn*, two other osteoblast-specific genes. The expression of these three genes was significantly increased when either *Pbx1-d* or shRNA was expressed as compared with cells transduced with EVs (Figure 1d). Overall, these results demonstrate that *Pbx1-d* expression is functionally equivalent to knocking down *Pbx1* expression in accelerating MC3T3-E1 cell differentiation.

Pbx1-d attenuates Sox3 transcription

To further explore *Pbx1-d* function, we compared the activity Sox3 promoter:luciferase constructs in MC3T3-E1 cells transduced with LV-*Pbx1-d*, shRNA *Pbx1* or EV. We confirmed that the two promoter constructs that contain a *Pbx1*-binding site resulted in a higher luciferase activity than the construct containing only the basal promoter (Figure 2a). More interestingly, LV-*Pbx1-d* or shRNA *Pbx1* transductions resulted in a significant lower luciferase activity of the constructs containing the *Pbx1*-binding site (Figures 2a and b). This showed that *Pbx1-d* has a lower transactivation capacity than the endogenous isoform.

Pbx1-d binding to the Sox3 promoter was investigated by electrophoretic mobility shift assay with nuclear extracts (NEs) prepared from MC3T3-E1 cells transduced with LV-*Pbx1-d*, shRNA *Pbx1*, *Pbx1-b* or EV. With titrated probe concentrations, NE from cells transduced with *Pbx1-b* resulted in consistently increased amount of probe/NE-specific complexes relative to the endogenous *Pbx1-b* in NE from cells transduced with LV-EV (Figures 3a and b). The small difference between LV-*Pbx1-b* and LV-EV suggests that near-saturating amount of endogenous *Pbx1-b* is bound to the *Sox3* promoter. *Pbx1-d* expression resulted in the opposite effect with a lower amount of probe/NE complexes as compared with LV-EV or LV-*Pbx1-b*, with the difference reaching significance with a nonsaturating amount of probe. The amount of probe/NE-specific complexes was equivalent between MC3T3-E1 cells transduced with LV-*Pbx1-d* and LV-shRNA *Pbx1*, which was lower than the probe complexes in NE from cells transduced with LV-EV (Figure 3c). This suggested that *Pbx1-d* competes with endogenous *Pbx1-b* for cofactors, such as Meis, that favor its binding to the *Pbx1*/Meis-binding site. We tested this hypothesis by preincubating NEs from LV-EV- or LV-*Pbx1-d*-transduced cells with anti-Meis or anti-*Pbx1* antibodies (Figure 3d). Anti-*Pbx1* resulted in a small decrease in the amount of probe/NE-specific complexes, as previously described.¹⁹ Anti-Meis also slightly decreased the amount of complexes with endogenous *Pbx1-b*, but the difference was greater in LV-*Pbx1-d*-transduced cells. This suggests that a *Pbx1*/Meis complex binds the Sox3 promoter and that *Pbx1-d* sequesters Meis. The reduction of Meis levels by the combined presence of *Pbx1-d* and anti-Meis antibody greatly reduced the amount of endogenous *Pbx1* bound to the probe in LV-*Pbx1-d*-transduced cells. These results strongly suggest that *Pbx1-d* does not bind to the *Sox3* promoter and functions as a dominant negative decreasing the transactivation function of endogenous *Pbx1-b*.

PBX3, whose expression pattern largely overlaps with PBX1, has a splice isoform whose expression is favored in leukemic cells, suggesting that a truncated protein can oppose the

function of the normal isoforms and lead to a pathogenic outcome.²⁰ In addition, two non-homeodomain cofactors have been shown to have critical roles in cell differentiation by preventing PBX1-HOX DNA binding. Zinc finger Pbx1 interacting protein interacts with *Pbx1-b* and prevents its binding to DNA in selected cell types, such as the embryonic genital tract.²¹ More directly related to the immune system, the prevention of *Pbx1* binding to DNA by human Pbx1 interacting protein is necessary for hematopoietic cell differentiation.^{22,23} These findings suggest that in addition to being a dominant negative, *Pbx1-d* may have its own regulatory function through the non-homeodomain pathways. Therefore, the association between the expression of *Pbx1-d* and lupus susceptibility may be mediated through the regulation of Pbx1/Hox/DNA-binding complexes achieved in a similar manner in other cell types by non-Pbx proteins. The contribution of this mechanism relative to a direct dominant-negative function will be directly tested in T-cell lines as well as primary T cells.

Pbx1-d expression increases CD44 expression in Jurkat T cells

Pbx1-d affects the intrinsic phenotypes of CD4⁺ T cells in the NZM2410 lupus model.³ We do not know at this point as to which genes *Pbx1* directly targets in T cells, making it impossible to assess the dominant-negative function of *Pbx1-d* in this cell type. We have previously shown that a hallmark of B6.*Sle1a1* T cells was the increased expression of activation markers, including CD44,^{1,2} which we confirmed here not only in the spleen but also in the gut-associated lymphoid tissue (Figure 4a). Epigenetic regulation has an important role in *Cd44* expression.²⁴ Because *Pbx1* transactivates gene expression through the recruitment of chromatin-remodeling factors,^{7,17} we postulated that *Cd44* could be one of its target genes. In addition, *Cd44* expression is directly regulated by Bcl-6,²⁵ and Bcl-6 has been recently identified as a direct target of Pbx1.²⁶ Collectively, these results indicated that *CD44* expression could be directly or indirectly regulated by *Pbx1-d*. In support of this hypothesis, *CD44* expression was increased in Jurkat T cells transduced with either *Pbx1-d* or *Pbx1* shRNA as compared with EV (Figure 4b). This suggests that *Cd44* expression is directly or indirectly repressed by *Pbx1* and that either *Pbx1* downregulation or dominant-negative Pbx1-d expression promotes *Cd44* expression.

MATERIALS AND METHODS

Cell culture

Newborn mouse calvarial-derived MC3T3-E1 subclone 14 preosteoblastic cells (ATCC, Manassas, VA, USA) were cultured in MEM medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, St Louis, MO, USA), 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin (Gibco Life Technologies, Grand Island, NY, USA) at 37 °C with 100% humidity and 5% CO₂. The complete medium was replaced every 2–3 days and confluent cells were subcultured through trypsinisation. MC3T3 cells were allowed to grow until they reached 80–90% confluence, approximately 2 days post plating. The media was then changed to osteoblast differentiation media containing 50 µg ml⁻¹ ascorbic acid and 10 mM β-glycerophosphate (Sigma Aldrich). Differentiated cells switch from a fibroblastic to a dense cobblestone morphology. Alkaline phosphatase activity was measured 12 days later with BCIP/ NBT substrate (Sigma Aldrich) after fixation with 10% neutral buffered formalin. Quantitative reverse transcriptase PCR analysis of osteoblast-specific genes was performed under optimized conditions with SYBR Green reagents (Life Technologies). The LV-GFP-Pbx1-d, LV-GFP-Pbx1-b and LV-GFP (EV) constructs have been previously described.³ The Pbx1 shRNA LV vector msPbx1-T2 was a kind gift from Dr Jane Lian (University of Massachusetts Medical School). A total of 100 p.f.u. per cell of each recombinant LV construct were mixed with culture media containing 4 µg ml⁻¹ polybrene, then were added to 1 × 10⁶ MC3T3-E1 cells cultured to approximately 70% confluent growth. After 24 h, the transduced cells were placed into

complete MEM medium. Transduction efficiency was 60–75% and resulted in stably expressed constructs. Transduction with the various constructs did not affect cell viability, growth or density. Jurkat T cells were transduced with Pbx1-d LV and Pbx1 shRNA LV as previously described.³ Reverse transcriptase PCR was performed on these cells with primers for *Cd44* (F: 5'-ACTTCACCCCAACAATCTTGA-3' and R: 5'-GTGGCTTGTTGCTTTTCAGT-3') and *Cd4* (F: 5'-GAACCTGGTGGTGATGAAAG-3' and R: 5'-CACCCCTCTGGATAAACCT-3').

Western blot analysis

Whole-cell lysates were prepared from MC3T3 cells 2 days after transduction with the Pbx1-d, Pbx1-b LV, Pbx1 shRNA LV or EV constructs. Western blot analysis was performed using a polyclonal anti-Pbx1 antibody (ab12001; Abcam, Cambridge, MA, USA) followed by detection with anti-rabbit horseradish peroxidase/ECL reagent as previously described.²⁷

Electrophoretic mobility shift assay

A probe was designed to span the Pbx1-binding site in the mouse *Sox3* promoter by homology to the human *SOX3* promoter.¹⁹ The probe was generated by annealing complementary sense (5'-ACGGCGAGCCTGTCAA TCACGAGGC-3') and anti-sense (5'-CGTGGGCCTCGTGATTGACAGGCTC-3') oligonucleotides. NEs from transduced MC3T3 cells were prepared 4 days after differentiation induction, with a Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The binding reactions with 10 µg of NE were carried out as previously described.²⁸ In some experiments, NEs were incubated with the ab12001 anti-Pbx1 or anti-Meis (sc-10596, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies for 20 min at room temperature prior to the addition of the probe. In competition assays, 100-fold molar excess of unlabeled competitor was included in the binding reaction.

Luciferase assay

Three segments of the *Sox3* promoter were cloned with the following primers: – 587/ +25: 5'-GAAGATCTGAAGATCTGTAGCGCACCTCCGTTATA CACT-3' and 5'-CCCAAGCTTATGCGTTCTCTCGAGCTGGT-3'; –138/ +25: 5'-GAAGATCTGTTGATTGGCCAGGACTCAT-3' and 5'-CCCAAGCTTATGCGTTCTCTCGAGCTGGT-3'; and –62/ + 25: 5'-GAAGATCTCAACCCTCCCGGATC TGA-3' and 5'-CCCAAGCTTCCCAAGCTTATGCGTTCTCTCGAGCTGGT-3'. *Bgl*II and *Hind*III restriction sites were inserted in the upstream and downstream primers, respectively, for cloning purposes. PCR products were individually inserted into a TK-luc⁺ PGL-4 basic reporter vector (Promega, Madison, WI, USA). The accuracy of each construct was verified by sequencing. The – 587/ + 25 and –138/ + 25, but not –62/ + 25, constructs contain a strong Pbx1-binding site.¹⁹ MC3T3-E1 cells expressing Pbx1-d or Pbx1 shRNA LV were transduced with 50 ng TK-luc⁺ vector or 1.6 µg individual *Sox3* promoter constructs. Cells were lysed 48 h later to measure luciferase activity as previously described.²⁸ Normalization was performed to the TK-luc empty construct.

Flow cytometry

Flow cytometry was performed on splenocytes and cells from mesenteric lymph nodes and Peyer's patches from 7-month-old B6.*Sle1a1* and B6 mice with anti-CD4-FITC (RM4–5) and anti-CD44-PE (IM7, both from eBioscience, San Diego, CA, USA) as previously described.³

Statistical analysis

Statistical analysis was performed using Prism software (Graphpad Software, La Jolla, CA, USA). Unless specified, graphs show means and s.e. Statistical significance was reached with *P*-values 0.05 in the two-tailed tests indicated in the figure legends.

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Figure 1.

Pbx1-d increases osteoblast differentiation in MC3T3 cells. **(a)** Western blot analysis showing the expression of endogenous Pbx1-b and LV-expressed Pbx1-d (top panel with two separate transductions) in transduced MC3T3 cells, as well as Pbx1-b over- and knocked-down expression in MC3T3 cells transfected with Pbx1-b LV or shRNA Pbx1 LV, respectively (bottom panel). **(b–d)** MC3T3 cells were transduced with LV vectors expressing either Pbx1-d, Pbx1 shRNA or EV and were analyzed 12 days after differentiation induction. **(b)** Representative image of alkaline phosphatase (ALP) activity in the absence (–) or presence (+) of β -glycerophosphate and ascorbic acid differentiation inducers. Each panel represents a confluent cell monolayer in a single well in which ALP⁺ cells are stained purple. **(c)** Image analysis of Alp activity. *P*-values correspond to Dunnett's multiple comparison tests. **(d)** Quantitative reverse transcriptase PCR analysis of *Alp*, *Bsp* and *Ocn* expression (*n* = 5 independent transductions). Values were normalized to uninduced EV-transduced cells (EV –). *P*-values correspond to Student's *t*-test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; and *****P* < 0.0001.

Figure 2.

Pbx1-d reduced the activity of the *Sox3* promoter. **(a)** Three *Sox3* promoter:luciferase constructs, two of which (–587/ + 25 and –138/ +25) including the Pbx1-binding site, as well as the luciferase construct alone (PGL4), were transduced in MC3T3-E1 cells transduced with either LV-Pbx1-d, Pbx1 shRNA or EV. Luciferase activity was normalized to the value obtained with the –62/ +25 construct. The graph showed the individual values obtained in three independent experiments with the bars showing the mean values. **(b)** Pooled luciferase activity obtained with the two Pbx1-binding site constructs for each cell type. *P*-values correspond to Dunnett's multiple comparison tests, ***P*<0.01.

Figure 3.

Pbx1-d does not bind to the *Sox3* promoter *in vitro*. (a) Representative electrophoretic mobility shift assay (EMSA) was carried out with a probe for the *Sox3* promoter containing a Pbx1-binding site with the conditions reported for each lane and NEs from MC3T3-E1 cells transduced LV-EV, Pbx1-d or Pbx1-b. The bands at the top show the specific probe/NE complex that was completely competed out by the cold probe (lane 3). The amount of hot probe was $1 \times$ (lanes 1–5), $0.5 \times$ (lanes 6–8) and $0.1 \times$ (lanes 9–11). The bands at the bottom show unbound free probe. (b) Band intensities of EMSA shown in (a), between the three types of NEs with graded amount of probe (mean and s.d., 3–5 samples per cell type and probe concentration). (c) NEs from cells transduced with either Pbx1-d or shRNA Pbx1 show decreased binding to the *Sox3* promoter. (d) NE were preincubated with either anti-Pbx1 or anti-Meis antibody, which decreased the amount of probe/Pbx1-specific complexes. The combined lack of DNA binding of Pbx1-d and the strong binding to Meis result in a greatly diminished amount probe/Pbx1 complexes in the LV-Pbx1-d-transduced cells. For (c, d), band intensities are indicated for each lane. The *P*-value corresponds to Student's *t*-test. **P* < 0.05.

Figure 4.

Increased CD44 expression by *Sle1a1* CD4⁺ T cells is induced by Pbx1-d expression in Jurkat T cells. (a) Representative histograms of CD44 staining in CD4⁺-gated cells in the spleen, mesenteric lymph node (MLN) and Peyer's patches (PP) of B6.*Sle1a1* (empty) and B6 (gray filled) mice. (b) *CD44* expression in Jurkat T cells, either transduced with EV, Pbx1-d or shRNA Pbx1 LV, normalized to *CD4* expression. The numbers indicate the *CD44/CD4* ratio for each sample.