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Role for Transcription Pax5A Factor in Maintaining Commitment to the B Cell Lineage by Selective Inhibition of Granulocyte-Macrophage Colony-Stimulating Factor Receptor Expression

Mark Y. Chiang and John G. Monroe

During early B lymphopoiesis, developing B cells maintain lineage commitment despite the local presence of myeloid lineage-promoting cytokines such as GM-CSF and IL-3. Previous observations suggest that the B cell-specific transcription factor Pax5A (paired box 5A transcription factor) plays a role in maintaining B cell lineage commitment by limiting expansion and survival of early IL-3/GM-CSF-dependent myeloid lineage cells. To define a mechanism by which Pax5A can exert these inhibitory effects on myeloid lineage differentiation, an inducible form of the Pax5A protein was expressed in the myeloid cell line FDC-P1. This cell line models myeloid progenitors in that it responds to the survival and growth-potentiating effects of IL-3 and GM-CSF. We observed that enforced expression of Pax5A selectively suppressed proliferation in response to GM-CSF, but not IL-3. This effect was associated with specific down-regulation of GM-CSFR α-chain, but not β-chain expression. These data provide a molecular mechanism to enforce commitment to the B cell lineage despite the presence of GM-CSF, a factor that has been shown to convert early developing B cells to the myeloid lineage. Furthermore, they indicate a role for B cell Pax5A expression in maintaining rather than directing commitment to the B cell lineage. The Journal of Immunology, 2001, 166: 6091–6098.

H emopoiesis is marked by plasticity, which is progressively lost as multipotential stem cells express new patterns of gene expression associated with stable commitment to specific hematopoietic lineages. For the B lymphocyte, early stages of differentiation are marked by expression of B lineage-restricted proteins, such as B220, CD19, CD79a (Igα), and CD79b (Igβ) (1–3). In addition, the early stages of B cell lineage development are defined by sequential and B cell lineage-restricted recombination of genes encoding the heavy chain of the B lymphocyte Ag receptor (1, 4).

Despite these B lineage-restricted events, there is increasing evidence to indicate potential for early stage B lymphocytes to differentiate along pathways leading to other hematopoietic lineages. Recent evidence argues that normally this plasticity is limited by the expression of B lineage-specific transcription factors such as the Paired Box 5A transcription factor (Pax5A)3 (5–7). These transcription factors presumably function either by directing differentiation to the B lineage or alternatively, by blocking pathways that allow divergence toward other lineages. The demonstrated plasticity of early pro-B cells lacking Pax5A expression (5, 6) as well as the Pax5A-dependent suppression of myeloid differentiation documented by our laboratory (7) argues that Pax5A functions by limiting divergence toward other lineages. In these studies, expression of Pax5A suppressed in vitro differentiation toward and along the myeloid lineage, as determined by morphology, flow cytometry, and responsiveness to growth factor, as well as the expression of a number of myeloid markers such as M-CSFR and myeloperoxidase (6). However, the molecular mechanisms by which Pax5A exerts these effects and thereby maintains commitment to the B cell lineage have not been directly addressed by these previous studies.

Pax5A is the major alternatively spliced isoform of Pax5, a member of the Pax family of transcription factors (8). It is first expressed immediately after B cell lineage commitment (3, 9) and continues to be expressed throughout B cell development, except in plasma cells (10). Pax5A−/− mice completely lack B220+ cells in the fetal liver, suggesting that Pax5A is required for B cell commitment (11). However, in the bone marrow, Pax5A−/− mice display a block somewhat later in B cell development, immediately after D to J, but before V to DJ rearrangement of the Ig heavy chain locus (11). Pax5A is also thought to up-regulate several B cell-specific genes that are first expressed during the early pro-B stage. These genes, which include VpreB1, λ5, blk, mb-1 (Igα), and CD19, encode both markers of early B cell lineage commitment as well as proteins that are necessary for the transition through the initial stages of B cell development in both the bone marrow and the fetal liver (12–14).

As discussed, differentiating B cells have the potential for divergence toward other hematopoietic lineages. In particular, developing B cells may be especially prone to divergence toward the myeloid lineage. In fact, recent evidence suggests that these two lineages may share a common bipotential precursor (15, 16). The mechanisms used by early B cells to limit divergence to the myeloid lineage are weak or can be disrupted by transformation, as...
many transformed pre-B cell lines and primary B cell lines spontaneously differentiate or can be induced to differentiate into myeloid cells (17, 18). Cytokines that induce these pre-B cells to convert to the myeloid lineage include myeloid growth factors such as M-CSF, GM-CSF, and IL-3 (18–22). For example, either IL-3 or GM-CSF can induce the pre-B cell line SPGM-1 to down-regulate expression of the B cell markers A5, VpreB, Mb-1, B29, PB76, and Igμ, and up-regulate the expression of the myeloid markers CD11b, F4/80, and M-CSFR (19). These cells also become myeloid-like morphologically, phagocytose latex beads, and adhere to plastic (19). Because developing B cells do not normally differentiate to the myeloid lineage, the myeloid lineage-converting effects of multiple cytokines present in the bone marrow must be regulated at critical checkpoints. The study reported in this work was designed to identify a mechanism by which Pax5A could limit the plasticity of developing B cells. Specifically, plasticity between the B lymphoid and myeloid lineages was targeted for study because of the apparently close developmental relationship between these two lineages.

Materials and Methods

Cell line culture

FDC-P1 cells and transfectants were cultured in medium containing IMDM, 10% FCS, 50 μM 2-ME, and 10% WEHI-3B cell supernatant as a source of IL-3. EML/Hyg and EML/Pax5 cells were cultured and induced toward myeloid differentiation, as described for previous studies (7).

Oligonucleotides

All oligonucleotides were prepared by the Nucleic Acid Facility at the University of Pennsylvania Cancer Center. The sequences of the primers of Pax5–12 and Pax5–16 are, respectively, 5′-GGGCGCGCGCATCGA TACCCTGCGCATTACAGG-3′ and 5′-GGGAATTCGGGCTGCAGGAGGTCCTTCCT-3′. The primers used to amplify GM-CSFR cDNA are 5′-GCGGCGACAGCGAGTATACGG-3′ and 5′-CTA GGGTCGGGAGGCTCTCCCT-3′. The sequence of the GM-CSFRα internal primer is 5′-TGGCTGAGCTCGAGGATGTAG-3′. The sequences of Pax5–17 and Pax5–18 are, respectively, 5′-GGGATCC ATTTCATTTTCTAGAAG-3′ and 5′-GGAAGCTTCTCTGGGTCGCTT AAGGCTGTCGG-3′.

Plasmid construction

To construct pAcPax5A-TR (Pax5A-TR, Pax5A/tamoxifen receptor fusion protein), a fragment containing the murine Pax5A cDNA flanked by a NoI site and a ClaI site on the 5′ end and a BgII site on the 3′ end was generated using PCR with the primers Pax5–12 and Pax5–16 and with pmBSAP-2 (BSAP, B cell-specific activation protein) (23) (M. Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) as the template. pmBSAP-2 contains the full-length murine Pax5A cDNA downstream of the CMV promoter and upstream of the SV40 polyadenylation signal. The resulting fragment was ligated into the NoI and BamHI sites of pEREHBD(BamHI/RJ) (T. Littlewood, Biochemistry of the Cell Nucleus Laboratory, Imperial Research Cancer Fund, London, U.K.), which contains the tamoxifen receptor CDNA, to generate pPax5ATR. The fragment containing the fusion product was then subcloned into the ClaI and BamHI sites of pCB76 (A. Kopp, Abteilung für Genetik, Medizinische Hochschule, Hannover, Germany) and pAcPol, respectively. The pAcPol poly was constructed by subcloning the BamHI to SalI region of pBluescript SK (Strategene, La Jolla, CA) into the BamHI and SalI sites of pAc-mEGR-1 (D. Liebermann, Temple University, Philadelphia, PA). To construct pAcPax5A-M, a fragment was generated using PCR with pmBSAP-2 as the template and with the primers Pax5–17 and Pax5–18. This fragment was then ligated into the EcoRI and HindIII sites of pmBSAP-2.

Generation of stable FDC-P1 transfectants expressing inducible and constitutive Pax5A

The stable cell lines FA and FABT were obtained by electroporation of FDC-P1 cells with the pAcPol and pAcPax5ATR constructs, respectively, followed by subcloning by limiting dilution. EMSA to measure Pax5ATR-binding activity was performed as previously described (7). The anti-estrogen receptor rabbit antiserum H-7 was generated against the first 20 amino acids of the hormone-binding domain of the estrogen receptor (A. Sewing, Imperial Research Cancer Fund).

As previously described (7), MIGR is an empty retroviral vector prepared by transiently transfecting the MIGR1 plasmid into the Bosc23 packaging cell line. It carries a long-terminal repeat that allows ectopic expression of integrated cDNA inserts, as well as a GFI marker from an internal ribosomal entry point. FDC-P1 cells were transduced as previously described (7) with the MIGR-Pax5A retrovirus containing the full-length Pax5A cDNA, or the MIGR parental retrovirus to generate Pax5A-expressing and non-expressing lines, respectively; both lack the tamoxifen receptor component. After transduction, the cells were cultured for 5 days and then cloned by sorting single green fluorescence protein (GFP)+ cells into individual wells of a 96-well plate using a FACScan (Becton Dickinson, Franklin Lakes, NJ). Single cell origin was confirmed by both visual inspection and limiting dilution analysis. Cell sorting for GFP-expressing cells was performed by the Flow Cytometry Center at the University of Pennsylvania Cancer Center. FDC-P1 cells transduced with the MIGR-Pax5A retrovirus were designated FMB cells, while FDC-P1 cells transduced with the parental MIGR retrovirus were designated FM cells.

Reporter assays

FABT cells and control FA cells were transiently transfected with pLucSCD19 (M. Busslinger, Research Institute of Molecular Pathology) and the SV-β-galactosidase control vector (Promega, Madison, WI). Luciferase and β-galactosidase activities were assayed according to the Dual-Light protocol (Tropix, Medford, MA). FDC-P1 cells were electroporated with various amounts of pmBSAP-M, SV-β-galactosidase control vector, and (PU.1)LBkCAT or LBkCAT (M. Atchison, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA). To standardize the total amount of electroporated DNA, appropriate amounts of CMV-2L5 plasmid were added. Lysate was harvested with Reporter Lysis Buffer (Promega) and assayed for chloramphenicol acetyltransferase (CAT) according to the manufacturer’s instructions. Lysate was also harvested with 50 μl of Galacto-Light lysis buffer (Tropix) and assayed for β-galactosidase activity according to manufacturer’s instructions.

Proliferation assays

FA and FABT cells were cultured in the presence or absence of 150 nM 5-hydroxytamoxifen (OHT) for 1–3 days, then recultured in IL-3-free medium for 24 h, and then plated at 2.5 × 104 cells/ml in 96 flat well plates for 24 h at various concentrations of murine IL-3 (mIL-3) (R&D Systems, Minneapolis, MN) or mGM-CSF (Novartis, Vienna, Austria) before being pulsed with 0.5 μCi [3H]thymidine. PBS/10% FCS was used to dilute the mIL-3 or mGM-CSF. DNA was harvested using a PHD cell harvester (Cambridge Technology, Watertown, MA), and incorporated [3H]thymidine was determined by liquid scintillation.

Analysis of α-chain and common β-chain expression of the GM-CSFR

FA and FABT cells cultured with OHT for 2.5–3 days were rested in IL-3–free medium for 24 h, washed with cold FACS buff (PBS, 2% FCS, 0.1% sodium azide), and incubated with hamster anti-mouse common β-chain (IL-3Rβ, AIC2B; Medical and Biological Laboratories, Nagoya, Japan). Biotinylated and affinity-purified anti-hamster (H+L) Ab (Vector Laboratories, Burlingame, CA) and streptavidin-PE (Becton Dickinson) were used as secondary and tertiary reagents, respectively. Flow cytometry was performed using a FACScan (Becton Dickinson). Semiquantitative RT-PCR was performed as described (24) to assay for GM-CSFR α- and β-chain expression (2). An internal G-CSFR α-chain primer and the 3′ β-chain primer were used as probes for Southern blotting.

Results

As already discussed, our previous study (7) and those using the Pax5A-deficient model (5, 6) have not directly addressed how expression of Pax5A limits lineage plasticity and contributes to maintaining commitment to the B lymphoid lineage. However, our studies in the EML stem cell line model and in primary bone marrow-derived stem cells have indicated that Pax5A expression negatively affects myeloid differentiation at a stage intermediate between IL-3- and GM-CSF-dependent stages (7). It has been well established that IL-3 can regulate early myeloid differentiation, whereas GM-CSF regulates the differentiation and expansion of later stage myeloid cells. The inability of EML cells to generate GM-CSF-responsive colonies under conditions of enforced Pax5A
expression suggests either direct effects of Pax5A expression on the signaling by these two growth-factor receptors or alternatively, inability of progenitors to differentiate to the GM-CSF-responsive stage. These results prompted us to consider that a mechanism by which Pax5A could limit conversion of early B cells to the myeloid lineage despite the myeloid potentiation effects of these cytokines in the bone marrow could be through disrupting signaling through one or both of these receptor systems.

Our previous studies could not address the mechanism of these effects because the cell populations were heterogeneous with respect to their differentiation stage and lineage commitment. Furthermore, these studies could not differentiate effects of Pax5A on cellular differentiation vs proliferation. Therefore, to directly analyze the effect of Pax5A expression on IL-3- and GM-CSF-dependent myeloid cell responses, we chose to switch to a homogenous, nondifferentiating myeloid cell line. The FDC-P1 myeloid cell line was specifically chosen for these studies because it was able to proliferate in culture in response to either exogenous IL-3 or GM-CSF (25, 26).

FDC-P1 is a Mac-1+/Mac-2+/Mac-3+/F4/80+/Thy-1.2+ nonleukemic cell line derived from long-term bone marrow culture in the presence of IL-3-containing medium. This cell line proliferates, but does not differentiate in response to either IL-3 or GM-CSF (25, 26). Because this cell line does not require stimulation with IL-3 to acquire GM-CSF responsiveness, it models the intermediate stage of myeloid development, in which our previous studies identified Pax5A-mediated suppressive effects.

Establishment of FDC-P1 expressing an inducible Pax5A

A plasmid allowing constitutive expression of Pax5A-TR was stably transfected into FDC-P1. Pax5A-TR was expressed as a tamoxifen receptor fusion protein allowing us to regulate the activity of the constitutively expressed protein by supplementing or omitting OHT in the culture medium, which regulates transition from a sequestered to a free and, therefore, active state. A Pax5A-TR-producing line (FABT) was generated and cloned by limiting dilution along with a control plasmid-transfected, nonproducing line (FA). Five clones of each were saved for further analysis. Similar results were obtained for all of the clones. However, only results from FABT and FA are shown in this work.

To determine differential and inducible expression of Pax5A-TR in FABT and FA cell lines, an EMSA of nuclear lysates from each line was performed using a labeled probe derived from the Pax5A-binding region of the gene for CD19 (Fig. 1A) (23, 27). In FDC-P1 lysates, the Pax5A-TR (see arrow “Pax5A-TR,” lanes 1 and 2, Fig. 1A) was obscured by a nonspecific band present in both Pax5A-TR-transfected and control cells. However, direct verification of expression of the Pax5A-TR fusion protein in the FABT, but not the FA line was accomplished by demonstrating an anti-estrogen receptor-induced supershift in the FABT, but not the FA cell line (lanes 2 and 4, respectively). Interestingly, the interfering band observed in the FDC-P1 transfectants was not observed in the B cell lines Bal-17 and Wehi-231. We also detected a similar migrating band that was observed in only the Pax5A-TR transfectants of these cells and was supershifted by Abs to Pax5A. A more rapidly migrating complex present in lysates from FABT but not FA cells (see arrow “dPax5A-TR,” lanes 1 and 2; Fig. 1A) is believed to be a degradation product of Pax5A-TR because an Ab directed to the Pax5A DNA-binding paired domain blocked formation of this complex (unpublished data). Importantly, none of these bands nor the supershifted complexes were observed in the FA line transfected with the control plasmid only (Fig. 1A, lanes 3 and 4). Finally, under these conditions of probe excess, we did not observe overexpression of the fusion protein in the transfected cells as compared with the level of expression of Pax5A in a pro-B cell line (unpublished data).

To determine whether Pax5A-TR was functional and inducible by OHT, FABT and control FA cells were transiently transfected with a luciferase reporter construct pLucSCD19. This reporter contains the cDNA for luciferase coupled to two binding sites for Pax5A within the context of a 1.3-kb fragment of the human CD19 promoter. A representative assay of reporter activity for the inducible Pax5A-expressing and control clones in the presence or absence of OHT is depicted in Fig. 1B. Addition of OHT to the medium increased luciferase activity in FABT cells by 121% (lanes 1 and 2, Fig. 1B). This inducible activity was reproducible and typical of Pax5A transactivation (unpublished data, 1999). No inducible activity was observed in the FA cell line. Activity in the absence of OHT was detectably higher in the FABT than in the control FA cells, suggesting a small degree of unregulated Pax5A activity. These results document differential expression of Pax5A-TR in the FABT and FA cells and further establish that while there may be a small degree of unregulated Pax5A activity, we clearly can inducibly activate the Pax5A expressed in the FABT cells.

Pax5A inhibits the growth response to GM-CSF, but not to IL-3

To determine whether the expression of Pax5A differentially affected GM-CSF- and IL-3-mediated growth responses, we added OHT to FABT cells cultured with increasing amounts of GM-CSF or IL-3. The OHT induction period was studied over 1–3 days. When cultured with IL-3, neither FABT nor FA cell proliferative responses were affected by OHT (Fig. 1C, top left and right panels, respectively). In contrast, GM-CSF-dependent proliferation was markedly reduced in OHT-treated FABT, but not FA cells (Fig. 1C, bottom left and right panels, respectively). Notably, FABT proliferation as determined by [3H]thymidine incorporation in the presence of GM-CSF, but not IL-3, was reduced 5-fold in the absence of OHT when compared with FA cells with or without OHT. This effect could be due to the basal level of Pax5A-TR transcriptional activity in the absence of OHT, as suggested by studies shown in Fig. 1B. Nevertheless, Pax5A induction clearly affected the growth response to GM-CSF, but not to IL-3. The results depicted in Fig. 1C are unlikely due to an integration site artifact, because such an event would have affected the response independently of OHT treatment. Moreover, in studies not shown in this work, similar results were found using each of the other independently generated FABT cell clones, making it even more unlikely that the Pax5A-mediated inhibition of GM-CSF proliferation is due to an integration site artifact.

Pax5A inhibition of GM-CSF-dependent proliferation occurs in the absence of the tamoxifen receptor

To rule out the possibility that the tamoxifen receptor domain fused to the end of Pax5A in the Pax5A-TR fusion protein was responsible for the ability of Pax5A-TR to suppress GM-CSF-dependent proliferation, FDC-P1 cells were transduced with MIGR-Pax5A, a retrovirus containing the full-length Pax5A cDNA, or MIGR, the parental retrovirus, as the negative control. The MIGR-Pax5A retrovirus expresses Pax5A from the retroviral long-terminal repeat as well as the GFP marker from an internal ribosomal entry site, as described previously (7). Thus, cells that express the GFP marker coexpress Pax5A.

Transduced cells identified by GFP expression were sorted by flow cytometry to yield single cell clones, as confirmed both visually and by limiting dilution analysis. FMB-17, FMB-22, and FMB-24 are FDC-P1 clones transduced with the MIGR-Pax5A retrovirus, whereas FM-2 and FM-3 are FDC-P1 clones transduced...
with the parental MIGR retrovirus. In response to IL-3, proliferation of the Pax5A-expressing FMB clones is comparable with the proliferation observed for the nonexpressing FM clones (Fig. 2). However, in response to GM-CSF, the proliferation of each of the FMB cells was reduced by 80–99% relative to the proliferation of the FM clones.

To rule out the unlikely possibility that the process of retroviral transduction (such as GFP expression or expression of viral RNA) negatively affects the ability of FDC-P1 cells to respond to GM-CSF, the proliferation of the parental FDC-P1 cell line was measured (Fig. 2). As expected, this level of proliferation was comparable with the level of proliferation of the FM cells, and, therefore, it is unlikely that the process of retroviral transduction is responsible for the impaired ability of these cells to proliferate in response to GM-CSF. For these results, we can conclude that the differential responses observed between FABT and FA cells treated with OHT are not an anomaly of the individual FDC-P1 clones in either system.

Pax5A expression affects assembly and expression of the GM-CSFR

The results to this point are consistent with a selective inhibitory effect of Pax5A on GM-CSFR, but not IL-3R activity. The GM-CSFR is composed of the specific α-chain and the common β-chain. The β-chain is shared with one of the IL-3Rs (28, 29). To further explore our observations, we considered the possibility that the α-chain or the common β-chain of the GM-CSFR may be down-regulated by Pax5A. Down-regulation of the α-chain might be predicted because Pax5A inhibits the function of PU.1. PU.1 is a transcriptional activator of the GM-CSFRα protein gene (30, 31). However, effects on expression of either component of the GM-CSFR would be predicted to alter cellular responses to this cytokine. To evaluate the effect of expression of Pax5A on the expression of the GM-CSFR, Pax5A activity was again induced in FABT
cells by OHT treatment. Following treatment for 48 h, we analyzed the expression of the common β-chain of the GM-CSFR. Fig. 3, A–D, shows a comparison of the β-chain expression on the surface of FABT and FA cells cultured in the presence or absence of OHT. Expression under each condition for both cells was identical, indicating that Pax5A activity did not affect level of β-chain expression. This result is consistent with unaffected IL-3 responses in the presence of Pax5A (Figs. 1 and 2), as the common β-chain is expressed by IL-3R.

However, very different results were obtained for analyses of GM-CSFR α-chain expression in the presence of Pax5A activity. Because reagents that would permit quantitative analysis of α-chain protein expression by flow cytometry are not available and the effects of Pax5A inhibition were likely mediated at the transcriptional level, we assessed relative levels of GM-CSFR α-chain expression by semiquantitative RT-PCR. As shown in Fig. 3 E, FABT cells expressed equivalent amounts of GM-CSFR α-chain transcripts in the presence or absence of OHT (lanes 1–3 compared with lanes 4–6, Fig. 3E). However, Pax5A-TR-expressing FABT cells showed a marked reduction in expression of GM-CSFR α-chain transcripts when cultured in the presence of OHT (compare lanes 7–9 with OHT-treated cells, lanes 10–12, Fig. 3E).

Normalization of the GM-CSFR α-chain message levels to the β-actin message levels from the same cDNA samples (Fig. 3E, lanes 13–24) allowed us to determine that OHT induction of Pax5A activity resulted in a 80–90% reduction in GM-CSFR α-chain expression relative to cells in the absence of OHT. This result is consistent with unaffected IL-3 responses in the presence of Pax5A (Figs. 1C and 2), as the common β-chain is expressed by IL-3R.

Pax5A inhibits the expression of the α-chain of the GM-CSFR in the multiprogenitor EML cells

We had previously shown that GM-CSF-dependent colony formation and cell development from both bone marrow stem cells and the multipotential cell line EML were inhibited by Pax5A (7). These previous results using primary cells and another cell line model are consistent with the effects of Pax5A expression shown in this study on GM-CSFR α-chain expression. Nevertheless, to formally eliminate the possibility that the Pax5A-mediated reduction of GM-CSFRα mRNA levels was unique to the FDC-P1 cell line, we evaluated the effects of Pax5A expression on GM-CSFR α-chain expression in the EML cell model used for our previous studies. EML cells are multipotent progenitor cells immortalized...
by a retinoic acid dominant-negative protein and differentiate along the myeloid pathway to the CFU/granulocyte-macrophage (CFU-GM) stage in the presence of IL-3 and retinoic acid (32). EML cells expressing Pax5A (EML/Pax5A) and EML cells expressing the control vector (EML/Hyg) were induced to undergo myeloid differentiation with IL-3 and retinoic acid for 3 days, as previously described (7). GM-CSF-dependent proliferation was markedly impaired in the EML cells expressing Pax5A, as measured in the CFU-GM assay (Fig. 4A). GM-CSF-α-chain levels were measured by RT-PCR (Fig. 4B). Consistent with our results in the FDC-P1 model, we observed that the EML/Pax5A clones described previously (7) express GM-CSFR α-chain at 27–35% the levels of GM-CSFR α-chain message found in the EML/Hyg clones (Fig. 4C). Consistent results were found in other experiments. These results unequivocally establish that the suppression of GM-CSFR α-chain expression by Pax5A is not unique to the FDC-P1 cells, but can be generalized to other myeloid cells.

Discussion

Pax5A has been implicated in directing commitment of multipotential progenitors to the B lymphoid lineage. Indeed, the abrogation of B cell development secondary to Pax5 loss, the positive regulatory function of Pax5A in the expression of numerous B cell genes, and the expression of this gene specifically at the earliest stages of B cell development would seemingly support this role. However, recent studies suggest a different role for Pax5A in B cell development (3). First, Pax5A expression cannot be detected in multipotent progenitors even with RT-PCR (3, 9), suggesting that Pax5A would not even be present to direct these cells to the B cell lineage. Second, enforced expression of physiologic levels of Pax5A in primary and long-term cultured progenitor cells and myeloid cells was shown to have minimal effect on directing differentiation to the B cell lineage (7, 33). Rather, we found that Pax5A blocked differentiation and survival of developing myeloid progenitors (7). These results suggest a role for Pax5A in limiting the plasticity between the myeloid and B lymphoid lineages at early stages of B cell development. Other recent studies have shown that pro-B cells deficient for Pax5A expression can differentiate into a variety of hemopoietic lineages, including those of the myeloid lineage (5, 6). Together, these results argue that Pax5A does not function to initiate cells into the B cell lineage, but more accurately, functions to maintain commitment to that lineage by suppressing divergence along alternative pathways until commitment is more firmly established. The studies described in this work were designed to investigate the mechanism by which Pax5A suppresses divergence with a specific focus on the myeloid lineage.

In this study, we show that the ectopic expression of Pax5A inhibits GM-CSFR-mediated proliferation, but not IL-3-mediated proliferation, and link this observation to a selective down-regulation of the GM-CSFR α-chain vs the GM-CSFR β-chain. We have demonstrated these results with both inducible and noninducible Pax5A expression constructs, thus controlling for site-integration artifact, clonal artifact, and artifact associated with using the tamoxifen receptor fusion protein. We have also demonstrated these results in both the FDC-P1 cell line and the EML cell line, thus controlling for peculiarities of cell lines and interpretation difficulties inherent to a differentiating cell line. Thus, we feel confident that our results are unlikely artificial and can be generalized broadly to myeloid cells. Furthermore, our results are consistent with recent studies that show that Pax5A inhibits the function of PU.1, a transcriptional activator of the GM-CSFRα gene that is essential for GM-CSF-, but not IL-3-dependent proliferation (30, 34, 35).

Although we have previously observed in vitro arrest of myeloid cell differentiation in the presence of Pax5A (7) and in this study show a negative effect on GM-CSF-mediated proliferation of myeloid cells, the direct effects of this transcription factor on myeloid development and proliferation are probably not significant, as there appear to be other redundant receptor systems in vivo that can substitute for the GM-CSF to support differentiation of cells committed to the myeloid lineage. This redundancy is evident by the fact that GM-CSF−/− mice show no steady state defect in myelopoiesis (36) despite the well-known effect of this cytokine in supporting myeloid differentiation in vitro. Rather, we believe the relevance of our findings to relate to the ability of GM-CSF to convert pre-B cells to the myeloid lineage (18, 19). Specifically, down-regulation of the GM-CSFR by endogenous Pax5A would

![FIGURE 4](http://www.jimmunol.org/) Pax5A suppresses GM-CSF-dependent proliferation and GM-CSFRα expression in the myeloid progenitor EML cell line. A. EML/Pax5A CFU-GM colonies compared with EML/Hyg colonies. Pax5A-expressing EML cells (EML/Pax5A) and control EML cells (EML/Hyg) were induced with 10% IL-3 containing supernatant from WEHI-3B cells and 10 μM all-trans retinoic acid for 3 days and then replated in 1% methylcellulose with 250 U/ml GM-CSF at 1.5 × 10⁵ cells per 3.5 cm plate. B. Semiquantitative RT-PCR performed on equal numbers of viable cells from the Pax5A-expressing and nonexpressing colonies to determine their relative level of GM-CSFRα mRNA expression. C. GM-CSFRα mRNA levels normalized to β-actin mRNA levels (at 24 PCR cycles) and plotted over the indicated number of PCR cycles.
provide a mechanism to prevent pre-B cells from converting to the myeloid lineage in response to local levels of GM-CSF.

Our data showing that Pax5A suppresses GM-CSF/Rα expression appear to conflict with data from recent studies (6), in which RT-PCR analysis revealed GM-CSF/Rα expression in both Pax5A-expressing and deficient pre-B cells. There are several possible explanations that could resolve this apparent conflict. First, the detection of GM-CSF/Rα expression in this previous report was not quantitative. When Pax5A/Rα–/– pro-B cell lines were in fact directly compared with similar lines ectopically expressing Pax5A, there is evidence for decreased expression of GM-CSF/Rα message associated with Pax5A expression (6). Second, the concentration of Pax5A-binding activity associated with these pre-B cells may be different from our FDC-P1 cells, although our EMSA studies have failed to detect significant differences when compared with established B cell lines (unpublished data, 1999). Third, the environmental conditions in which GM-CSF/Rα was measured were different. Specifically, these previous studies did not use IL-3 in the medium and cocultured the Pax5A/Rα–/– pro-B cells in the presence of ST2 stromal cells. ST2 cells have been shown to permit GM-CSF to convert pre-B cells to the myeloid lineage, despite presumed expression of Pax5A (37). Thus, bone marrow microenvironmental factors may modulate the inhibitory effect of Pax5A.

Studies are now underway to determine how Pax5A may reduce GM-CSF/Rα expression. PU.1 is a transcription factor expressed in pluripotent stem cells and myeloid and B lymphoid cells. It appears to be a myeloid lineage-determining gene, as enforced expression of PU.1 converts transformed stem cells into myeloid lineage cells. It is also thought to positively regulate the GM-CSF/Rα gene, which may explain why the absence of PU.1 impairs GM-CSF–, but not IL-3-mediated proliferation (34, 35). Thus, an attractive hypothesis is that Pax5A may inhibit PU.1 function and consequently shut down GM-CSF/Rα expression. This hypothesis is supported by recent studies of ST23 cells (30) that have shown that Pax5A and PU.1 associate directly and that this association results in inhibition of the transcriptional activity of PU.1. Our preliminary results confirm that this Pax5A-mediated inhibition of PU.1 translates to the F-DCP1 cell line in transient transfections and that this inhibition does not appear to involve effects on PU.1 expression or binding (unpublished data). Thus, the inhibition appears to act posttranslationally at the level of the PU.1 transcription factor level and may be a complicated dynamic event, involving multiple regulatory molecules. The expression of PU.1 in developing B cells appears to be at odds with its myeloid lineage-determining functions (38) and its ability to up-regulate the expression of the GM-CSF/Rα α-chain (39). Demonstration that Pax5A inhibits PU.1 in these cells would provide a reasonable explanation for this theoretical dilemma of how B cell lineage commitment is maintained in the presence of PU.1.

Given the complexity of hematopoiesis, it would not be surprising if Pax5A repression of myeloid potential is also mediated through effects on M-CSF, as argued by other studies (6). M-CSF is a growth-factor receptor gene that is expressed at a later stage in myeloid development beyond that represented by the FDC-P1 cell. Like GM-CSF/Rα, M-CSF appears to convert B-lineage cells toward the myeloid lineage when activated (22). Thus, some mechanism must be in place to block M-CSF-mediated conversion of early B cells to the myeloid lineage. Given that M-CSF appears to be regulated by PU.1 (34, 35, 40), an attractive hypothesis is that during B cell development, Pax5A inhibits PU.1, which then reduces expression of both M-CSF and GM-CSF. Future studies need to be done in cells further differentiated than F-DCP1 cells to see whether Pax5A actually blocks expression of M-CSF/Rα gene quantitatively and functionally. Such findings would suggest secondary or redundant mechanisms by which Pax5A may maintain B lineage commitment.

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References

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