Biphenotypic B-lymphoid/myeloid cells expressing low levels of Pax5: potential targets of BAL development

Szandor Simmons,1 Marko Knoll,1 Christopher Drewell,1 Ingrid Wolf,1 Hans-Joachim Mollenkopf,1 Corinne Bouquet,1 and Fritz Melchers1

1Lymphocyte Development Group, Max Planck Institute for Infection Biology, Berlin, Germany

The expression of Pax5 commits common lymphoid progenitor cells to B-lymphoid lineage differentiation. Little is known of possible variations in the levels of Pax5 expression and their influences on hematopoietic development. We have developed a retroviral transduction system that allows for the study of possible intermediate stages of this commitment by controlling the levels of Pax5 expressed in Pax5-deficient progenitors in vitro and in vivo. Retroviral transduction of Pax5-deficient pro-/pre-B cell lines with a doxycycline-inducible (TetON) form of the human Pax5 (huPax5) gene yielded cell clones that could be induced to different levels of huPax5 expression. Clones inducible to high levels developed B220+/CD19+/IgM+ B cells, while clones with low levels differentiated to B220+/CD19−/CD11b−/Gr-1− B-lymphoid/myeloid biphenotypic cells in vitro and in vivo. Microarray analyses of genes expressed at these lower levels of huPax5 identified C/ebpα, C/ebpβ, Pu.1, Csf1r, Csf2r, and Gata-3 as myeloid-related genes selectively expressed in the pro-/pre-B cells that can develop under myeloid/lymphoid conditions to biphenotypic cells. Therefore, reduced expression of huPax5 during the induction of early lymphoid progenitors to B-lineage–committed cells can fix this cellular development at a stage that has previously been seen during embryonic development and in acute lymphoblastic lymphoma–like biphenotypic acute leukemias. (Blood. 2012;120(18):3688-3698)

Introduction

During hematopoiesis from pluripotent hematopoietic stem cells, the transcription factor Pax5 is selectively expressed in B-lymphoid lineage cells. Pax5 directs the signaling component of the B-cell receptor (BcR; Ig), Igα, the costimulatory receptor CD19, and the signaling adaptor Blk.1,2 At the same time, Pax5 represses the multilineage and homing potential of the multilineage-primed Pax5+−/− pro-/pre-B cells by shutting down the expression of T lineage (eg, Notch-1),3 NK lineage (eg, 2B4, CD244),4 and myeloid cell lineage directed (eg, Csfr1, c-fms),5 B-lymphoid–inappropriate signaling genes.6,8 Furthermore, Pax5 represses expression of Flt3, a tyrosine kinase receptor that is required to develop common lymphoid progenitors (CLPs) from pluripotent hematopoietic stem cells.9 When Pax5 expression is turned off at the transition from B cells to plasma cells, some of the inappropriate gene expression programs are reactivated,8 as it happens when Pax5 is inactivated by cre-recombinase–mediated deletion in mature B cells.10

Pax5-deficient mice are blocked in early B-cell development11 at a B220+/CD19+/Fli1+/− c-kit+/IgM− pro-/pre-B–like state of differentiation11 while the development of all other hematopoietic lineages is unaltered. Cloned Pax5−−/− cells show remarkable flexibility of hematopoietic developmental choices. As CLP–similar, lymphoid-primed cells, they can be induced in vitro to precursor and immature T cells, to natural killer (NK) cells, and to different types of myeloid cells.4 They can also be transplanted into Rag-1− or Rag-2−–deficient, severe combined immunodeficient recipient mice and will populate all thymocyte, mature T-cell, and NK-cell compartments within 3 weeks, while all myeloid cell compartments are filled within 2 months.12 Unlike wild-type pro-B-I cells, Pax5−−/− pro-/pre-B cells home to the bone marrow from where they can be reisolated and repropagated in vitro.13 Hence, Pax5−−/− pro-/pre-B cells maintain self-renewal, multipotency, and homing potential for many successive transplantations.

Pax5 can play a divergent role in lymphomagenesis. Different groups have identified the PAX5 gene in some B-cell non-Hodgkin lymphomas. The coding regions of the PAX5 gene are translocated to, and its expression controlled by, regulatory elements of the IgH locus.14,15 Frequent somatic mutations in the PAX5 locus leading to haploinsufficiency and hypomorphic alleles or complete loss of function can be observed in paediatric acute lymphoblastic lymphomas (ALL).16-18 In addition, conditional deletion of PAX5 in CD19-CRE;Pax5−/− mice led to the development of B-progenitor lymphomas,19 whereas Pax5−/− heterozygous mice never develop malignancies.20 Restoration of Pax5 expression in Pax5−−/− mice and pro-/pre-B cells has been shown to repress B-lymphoid–inappropriate gene expression, and to promote development of B lymphocytes.20 To rescue Pax5 expression and B-cell development, we insert here the huPax5 gene into a retroviral transactivator (rtTA)–dependent TetON-expression vector21 that allows doxycycline-inducible huPax5 expression in Pax5−−/− pro-/pre-B cells. Retrovirally transduced cells are then cloned to generate lines that express huPax5 at different levels because different sites of insertion into the genome restrict retroviral gene expression to different extents.22 This allows us to investigate the influence of graded concentrations of huPax5
on molecular and cellular changes induced by huPax5 expression from a primed lymphoid-CLP–like to a B lymphoid–committed state. At low expression levels of huPax5, a B-lymphoid/myeloid biphenotypic state can be stabilized in appropriately doxycycline-dosed cells in vitro and in vivo.

Methods

Mice

Pax5−/−/Ly5.2− mice, a kind gift of M. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria), and Rag-2−/−/Ly5.1− mice were bred in the laboratory animal facility of the Max Planck Institute for Infection Biology (Berlin, Germany) under specific pathogen-free (SPF) conditions. All experiments complied with national regulations for the care and use of laboratory animals (protocol G0099/08, approved by the Landesamt für Gesundheit und Soziales, Berlin, Germany).

Cell culture

Two-week-old, bone marrow–derived Pax5−/− pro-/pre-B cells4−12 were grown in IMDM/2% FCS/0.03% primatone-RL (Quest) on H9251/-irradiated OP9 cells in IMDM/2% FCS/2% Flt3l/3% SCF to induce on molecular and cellular changes induced by huPax5 expression from a primed lymphoid-CLP–like to a B lymphoid–committed state. At low expression levels of huPax5, a B-lymphoid/myeloid biphenotypic state can be stabilized in appropriately doxycycline-dosed cells in vitro and in vivo.

In vivo differentiation of Pax5−/− pro-/pre-B cells into CD19+ pre-B and CD19+/B220+/CD11b+ biphenotypic cells

Six- to 12-week-old Rag-2−/− (Ly5.1−) mice were γ-irradiated (400 cGy) 24 hours before transplantation. Pax5−/− pro-/pre-B cells (5 × 10⁶ cells/mouse) were injected intravenously. In vivo differentiation of huPax5-TetON-transduced Pax5−/− pro-/pre-B cells into CD19+ pre-B- or CD19+/B220+/CD11b+ biphenotypic cells was induced in vivo 1 week after transplantation by 0.2 g/L doxycycline dissolved with 5% sucrose in acidified (pH 3.0) drinking water.

Antibodies and flow cytometry

If not stated otherwise, all anti–mouse antibodies were obtained from eBioscience Inc. Antibodies used for cell staining were Flt3-PE (A2F10), CD19-PE-Cy7 (1D3), biotinylated or allophycocyanin (APC)–conjugated B220 (RA3-6B2), CD11b-APC (M1/70), e-Kit-APC (ACK4), Gr-1–Pacific Blue (RB6-8C5). Streptavidin-Qdot605 (Molecular Probes) was used to visualize biotin-conjugated primary antibodies. Cells were incubated with anti–mouse CD16/CD32 followed by staining with the conjugated antibodies in PBS/2% FCS. Dead cell discrimination was performed by DAPI (Carl Roth). Analyses were done on a LSR-II flow cytometer (BD Biosciences) equipped with a 488-, 405-, 633-, and 355-nm laser.

Semi quantitative RT-PCR and quantitative real-time RT-PCR

Control- and Pax5−/− pro-/pre-B cells, induced in vitro by doxycycline to huPax5-dependent differentiation, were harvested and incubated for 45 minutes on a tissue-culture flask in IL-7/doxycycline to remove contaminating OP9 cells. RNA was prepared by using TRizol reagent (Invitrogen). For semi quantitative RT-PCR, cDNA was synthesized with random hexamer primers and Superscript III-RT (Invitrogen). PCRs were performed with a 4-fold dilution series (1:5) prepared of each cDNA sample.

Expression of mRNAs was quantitatively assessed by real-time RT-PCR using the Quantitect SYBR Green PCR Kit (QIAGEN) in a 7900HT fast real-Time PCR system (Applied Biosystems). Each sample was assayed in triplicate for every run, and results were normalized against GAPDH mRNA expression.

Immunoblotting

Control and Pax5−/− pro-/pre-B cells, induced in vitro by doxycycline to huPax5-dependent differentiation and OP9 separated, were lysed with modified RIPA buffer (25mM Tris-HCL,150mM NaCl, 1% NP40, 2% Triton X100, 2% CHAPS, 0.1% SDS, 1% protease inhibitor cocktail; Roche). Cell lysates (10 μg of protein/lane) were loaded on 4%-20% Mini-PROTEAN TGX gels (Bio-Rad), separated by electrophoresis, and blotted onto a nitrocellulose membrane. The membrane was blocked in 1× PBS with 0.1% Tween 20 (PBS-T) with 5% skim milk for 1 hour and incubated with a Pax5- or β-actin–specific antibody (Abcam, clone 12000, or GenScript) in 1× PBS-T with 5% skim milk overnight at 4°C. After wash in 1× PBS-T, membranes were incubated with a HRP-labeled polyclonal secondary anti–rabbit-specific Ab (Jackson ImmunoResearch Laboratories). Chemiluminescence was detected with the ECL Prime kit (PerkinElmer) using an LAS4000 imaging system (Fujifilm).

Results

Doxycycline-inducible Pax5-deficient pro-/pre-B-cell lines

Bone marrow–derived pro-/pre-B cells were first transfected with the retroviral vector pSR-rtTA21 expressing the gene for the TetON doxycycline-sensitive rtTA (Figure 1A). The rtTA-transduced Pax5−/− cells were thereafter transfected with a self-inactivating (SIN) retroviral vector containing the human form of the Pax5 (huPax5) gene under the control of a minimal CMV promoter containing an upstream Tet-responsive element (TRE) that binds rtTA (Figure 1A). When bound by doxycycline, rtTA can bind to
The retroviral huPax5-Tet-On expression system. huPax5 will be transcribed after binding of the doxycycline-dependent reverse transactivator rtTA-S2 to the transactivator-dependent TRE-element containing a pCMVmin. (B) Quantitative RT-PCR for huPax5 mRNA levels in doxycycline-induced Pax5−/− huPax5 pro-/pre-B-cell clone 4 before and 3 days after doxycycline administration (1000 ng/mL) in vitro. Each bar represents the mean ± SEM (error bars) of 3 individual experiments. (C) Western blot analysis with a Pax5-specific antibody of whole cellular lysates of huPax5 pro-/pre-B-cell clone 4 before and 3 days after doxycycline administration (1000 ng/mL) in vitro in comparison to wild-type pre-B-I cells and Pax5−/− #4. (D) Monitoring of huPax5-dependent CD19 and Flt3 expression by FACS analysis of huPax5 pro-/pre-B cells before and 3 days after doxycycline administration (1000 ng/mL) in vitro. Data are representative for 3 individual experiments.

Figure 1. Pax5−/− huPax5 pro-/pre-B-cell clone 4 expresses CD19 and down-regulates Flt3 surface expression on doxycycline-induced huPax5 expression in vitro. (A) The retroviral huPax5-Tet-On expression system. huPax5 will be transcribed after binding of the doxycycline-dependent reverse transactivator rtTA to the transactivator-dependent TRE-element containing a pCMVmin. (B) Quantitative RT-PCR for huPax5 mRNA levels in doxycycline-induced Pax5−/− huPax5 pro-/pre-B cell clone 4 before and 3 days after doxycycline administration (1000 ng/mL) in vitro. Each bar represents the mean ± SEM (error bars) of 3 individual experiments. (C) Western blot analysis with a Pax5-specific antibody of whole cellular lysates of Pax5−/− huPax5 pro-/pre-B-cell clone 4 before and 3 days after doxycycline administration (1000 ng/mL) in vitro in comparison to wild-type pre-B-I cells and Pax5−/− rtTA pro-/pre-B cells. β-actin-specific antibody was used as a loading control. (D) Monitoring of huPax5-dependent CD19 and Flt3 expression by FACS analysis of doxycycline-induced Pax5−/− huPax5 pro-/pre-B-cell clone 4. The numbers represent percentages of Flt3+ or CD19+ pro-/pre-B cells before and 3 days after doxycycline administration (1000 ng/mL) in vitro. Data are representative for 3 individual experiments.

Development of biphenotypic cells by graded huPax5 expression induced by different levels of doxycycline in vitro

Because normal levels of Pax5 induce the development to CD19+/ Flt3+ pre-B-I cells,2,5 we reasoned that submaximal levels of Pax5 might develop myeloid and lymphoid cells at intermediate stages of their development. We used clone 4, which is fully induced to development of biphenotypic cells by high concentrations (1000 ng/mL) of doxycycline, and applied varying doxycycline concentrations between 0 ng/mL and 1000 ng/mL in vitro on OP9/IL7 (Figure 3A).

Increasing concentrations of doxycycline led to increasing levels of huPax5 mRNA and protein levels (Figure 3B-C). Parallel to this doxycycline-dose–dependent up-regulation of huPax5 levels, CD19 mRNA and protein expression increased as well (Figure 3B-D). Above 200 ng/mL doxycycline, CD19 protein expression became detectable (Figure 3D). Maximal levels on the highest numbers of induced cells were reached at 1000 ng/mL doxycycline, which is comparable with the expression level of wild-type pre-B-I cells (Figure 3B-D). Doxycycline concentrations between 0 and 200 ng/mL induced huPax5 mRNA levels of not more than one-third of the wild-type control (Figure 3B), whereas huPax5 protein levels were approximately 100 times lower than the wild-type control when stimulated with 0-70 ng/mL doxycycline. The huPax5 protein levels increased slightly at 100-200 ng/mL doxycycline, but these levels of induced huPax5 did not lead to significant CD19 expression within 3 days of culture (Figure 3B-D). At that time, the microarray analyses described in the following paragraphs were done.
We have previously studied in vitro culture conditions for progenitors with myelopoietic and lymphopoietic potencies. From these experiments, we reasoned that SCF and Flt3l might favor survival and/or growth of differentiating B-lymphoid progenitors expressing different levels of huPax5.

After replating the cells at the same low doxycycline concentrations between 0 and 300 ng/mL on SCF/Flt3l/OP9 (Figure 3A), B220+/CD19−/CD11b− biphenotypic cells developed within the next 4 days (Figure 3E-F). For the next 19 days, these cells survived in SCF/Flt3l-containing cultures, remained biphenotypic but did not expand by proliferation (Figure 3E). Negative control Pax5−/− rTA cells did not express detectable levels of huPax5 or CD19 mRNA or protein (Figure 3B-D) and were not able to develop a B220+/CD19−/CD11b− phenotype (Figure 3E-F). By contrast, at high concentrations of doxycycline, clone 4 cells fully differentiated to B220+/CD19−/CD11b− pre-B-1 cells, and did not develop biphenotypic cells (Figure 3E).

Note that clone 4 used in these studies expressed low background levels of huPax5 in comparison to the Pax5−/− rTA control cells even in the absence of doxycycline (Figure 3B-C), apparently at sufficient levels to allow the huPax5-transduced cells to survive in the differentiation conditions (Figure 3E), and to exhibit the biphenotypic markers even in the absence of doxycycline induction (Figure 3F). These results suggest that very low levels of huPax5, well below those needed for the expression of CD19, appear to be sufficient to generate biphenotypic cells that can be stabilized in vitro on SCF/Flt3l/OP9.

Development of biphenotypic cells from Pax5-deficient progenitors expressing low levels of huPax5 after induction with high levels of doxycycline in vitro

Because we had found that the levels of uninduced or doxycycline-induced huPax5 expression appeared to determine the development of biphenotypic cells, we reasoned that clones 16 and 20 that expressed only low levels of Pax5 should be able to develop biphenotypic cells even at high concentrations of doxycycline in culture. The levels of huPax5 mRNA expression of clones 16 and 20 induced by doxycycline at high concentrations (1000 ng/mL, Figure 2A) were comparable with those in clone 4 cultured with 0 and 300 ng/mL (Figure 3B). Furthermore, Western blot analysis of huPax5 levels detected in clones 16 and 20 cultured under high doxycycline concentrations (Figure 2B) were comparable with those of clone 4 stimulated with low levels of doxycycline (Figure 3C).

These results suggest that submaximal huPax5 expression can be stabilized by retroviral integration at selected sites in the genome. We reasoned that such clones should be able to develop biphenotypic cells, even at high doxycycline concentrations.

To test this hypothesis, we performed an in vitro differentiation experiment of clones 16 and 20 cultured with high doxycycline
Figure 3. Pax5−/− huPax5 pro-/pre-B-cell clone 4 can be induced to develop into biphenotypic cells by graded huPax5 expression induced by different levels of doxycycline in vitro. (A) Experimental overview: Pax5−/− huPax5 pro-/pre-B-cell clone 4 was induced to express huPax5 by different levels of doxycycline (0-1000 ng/mL) in IL-7/OP9 for 3 days in vitro and was subsequently cultivated in SCF/Flt3l/OP9 with the same levels of doxycycline for 23 days. (B) Quantitative RT-PCR for huPax5 (i) and CD19 (ii) mRNA levels of Pax5−/− huPax5 pro-/pre-B-cell clone 4 induced by different levels of doxycycline (0-1000 ng/mL) in IL-7/OP9 in vitro. Results were normalized against GAPDH expression and plotted relative to control Pax5−/− rtTA pro-/pre-B cells. Each bar represents the mean ± SEM (error bars) of 3 individual experiments. (C) Western blot analysis with a Pax5-specific antibody of whole cellular lysates of Pax5−/− huPax5 pro-/pre-B-cell clone 4 before and 3 days after graded doxycycline administration (0-1000 ng/mL) in vitro in comparison to wild-type pre-B-I cells and Pax5−/− rtTA pro-/pre-B cells. β-actin–specific antibody was used as a loading control. (D) Representative FACS analysis (n = 3) of CD19 surface expression of Pax5−/− huPax5 pro-/pre-B-cell clone 4 induced by different levels of doxycycline (0-1000 ng/mL) in vitro. CD19 surface expression was detected at doxycycline levels more than 100 ng/mL. (E) Growth curves of Pax5−/− huPax5 pro-/pre-B-cell clone 4 induced to express huPax5 by different levels of doxycycline (0, 10, 300, and 1000 ng/mL) in IL-7/OP9 that were subsequently cultivated in SCF/Flt3l/OP9 (d0) at the same doxycycline concentrations for 23 days. (F) Representative FACS analysis (n = 3) of B220, CD19, and CD11b surface expression of Pax5−/− huPax5 pro-/pre-B-cell clone 4 cultivated for 4 days in SCF/Flt3l/OP9 at different levels of doxycycline (0, 10, 300, and 1000 ng/mL).
concentrations on SCF/Flt3/OP9 (Figure 2D). B220+/CD19−/CD11b+ biphenotypic cells developed from these clones within 4 days of differentiation (Figure 2E). Again they did not expand by proliferation, but survived for the next 19 days on SCF/Flt3/OP9 and remained biphenotypic (Figure 2F).

**Lack of in vitro proliferation of biphenotypic cells in cytokine environments favoring either myeloid, T-lymphoid, or B-lymphoid cells**

The development of biphenotypic cells from Pax5-deficient pro-/pre-B cells expressing low levels of huPax5 in vitro led us to the hypothesis that these biphenotypic cells may have the capacity to proliferate, and possibly differentiate in cytokine environments favoring either myeloid (ie, OP9/M-CSF, OP9/GM-CSF, or OP9/IL-3), T-lymphoid (ie, OP9-Δ1-1/Flt3/IL7), or B-lymphoid (ie, OP9/Flt3/IL-7) cell development.

To test this, we induced the development of biphenotypic cells from clone 4 (see conditions described in “Development of biphenotypic cells by graded huPax5 expression induced by different levels of doxycycline in vitro”). Biphenotypic cells were then replated into cytokine environments favoring either T-, B-lymphoid, or myeloid cell development, in the presence of 0, 10, and 300 ng/mL doxycycline. We monitored the proliferation and surface marker expression of the biphenotypic cells for the next 24 days of culture in these different conditions. As shown in supplemental Figure 2, we could only observe a proliferative expansion of cells in the presence of IL-7, which favors B-lymphoid (OP9) or T-lymphoid (OP9-Δ1-1) development. Under lymphoid culture conditions, biphenotypic cells proliferated as B220+ cells and lost their B220+/CD11b+ biphenotypic expression (not shown). In contrast, all conditions favoring myeloid cell development did not lead to a proliferative expansion of biphenotypic cells, or any other myeloid cell type (supplemental Figure 2).

In conclusion, we did not find tissue-culture conditions that would have allowed not only an increased survival, but a proliferative expansion of these biphenotypic cells, or of cells differentiating from them along the myeloid pathway of differentiation.

**In vivo induction of huPax5 in transplanted huPax5-transduced cell clones**

Because doxycycline can be fed with the drinking water, the induction of huPax5-transduced Pax5−/− pro-/pre-B-cell lines and clones (all Ly5.2+) along the B-lineage pathway of differentiation can also be monitored in vivo with cells transplanted into Rag-2−/−/Ly5.1+ recipient mice. One parameter of this differentiation in vivo is a loss of the homing properties of the transplanted Pax5−/− cells to the bone marrow. The feeding of mice with doxycycline can only be controlled with the highest concentrations, that is, at 0.2 g/L, which is equivalent in its inductive effect of full CD19 expression to 1000 ng/mL doxycycline in vitro.

Transplantation of clones 4, 16, and 20 (all Ly5.2+) into Rag-2−/−/Ly5.1+ mice and subsequent in vitro culture of bone marrow cells of the Rag-2−/−/Ly5.1+ recipients 16 weeks after transplantation did not recover any donor-derived cells as puromycin-resistant cells capable of in vitro proliferation on OP9/IL7 (not shown).

Transplantation of clones 16 or 20 (both Ly5.2+) into doxycycline-fed Rag-2−/−/Ly5.1+ mice (Figure 4A) allowed the isolation of puromycin-resistant cells from bone marrow 4 months after transplantation (not shown). The bone marrow of the reconstituted mice exhibited a dramatic expansion of the Ly5.2+ cell population of approximately 50% to 90% of all bone marrow lymphocytes, which is equivalent to approximately 5 × 10^5 to 1 × 10^6 total cells (Figure 4D-E). Interestingly, approximately 50% of the Ly5.2+ cell population was B220+/CD19−/CD11b+ (Figure 4D), hence, showed the biphenotype also seen in an in vitro differentiation experiment on OP9/SCF/Flt3 with high-dose doxycycline induction (Figure 2E).

From these experiments, we conclude that doxycycline-induced huPax5 expression from retroviral integration sites allowing only submaximal expression levels of huPax5 stabilizes a highly proliferative biphenotypic cell stage in vivo.

Our observation that the abundance of Pax5 is critical for the stabilization of biphenotypic cells in vivo offers the possibility that we can find in the bone marrow of Pax5−/− mice accumulated B220+/CD19−/CD11b+ cells. However, bone marrow of adult wild-type (Pax5+/+) and heterozygous (Pax5+/−) mice have almost no B220+/CD19−/CD11b+ biphenotypic cells (supplemental Figure 3). It should be noted that Pax5−/− and Pax5+/− mice develop comparable numbers of B220+/CD19+/CD11b− B lymphocytes in the bone marrow (supplemental Figure 3) and secondary lymphoid organs. Hence, Pax5 expression levels in progenitor and mature B lymphocytes of Pax5−/− mice are sufficient to induce CD19 expression. Because our results show that B220+/CD19−/CD11b+ biphenotypic cells develop from cells that express huPax5 at low levels that are insufficient to induce CD19 surface expression, it is reasonable to conclude that Pax5 levels in Pax5−/− mice are too high to allow the development and stabilization of biphenotypic cells.

**Concomitant transcription of myeloid- and lymphoid-related genes in Pax5−/−-rtTA-huPax5−transduced cells induced by low levels of doxycycline**

To study the repression and activation of genes induced by different levels of huPax5 through different concentrations of doxycycline, gene expression profiling was done either with Pax5−/− cells not transduced with huPax5, or with clone 4, transduced with huPax5 and induced to different levels of huPax5 by 0, 10, 300, or 1000 ng/mL doxycycline for 3 days in IL-7/OP9. Table 1 summarizes a collection of B-lymphocyte lineage-related genes previously detected in analyses performed on similar stages of B-cell development from Pax5−/− pro-/pre-B cells to more mature forms by Schebesta et al.6 The comparison of this previous analysis with the agreement in the detected genes (supplemental Table 1). Table 2 depicts characteristic examples, showing that CD19, CD79α, and Btk as B lineage–related genes are up-regulated, while Notch-1 as a T cell–related gene, Id2 as a NK lineage–related gene, and Csf1r as a myeloid lineage–related gene are down-regulated.

Importantly, however, the myeloid-related genes C/ebpα, C/ebpβ, Gata-3, FosL-2, and Csf2r remained up-regulated between 0 and 300 ng/mL doxycycline, only to be down-regulated at the highest levels doxycycline.
(1000 ng/mL) doxycycline concentration. These microarray analyses were confirmed by semiquantitative RT-PCR for C/ebp/H9251, C/ebp/H9254, Pu.1, Csf2r, Csf1r, Gata-3, Notch-1, CD19, CD79/H9251, and Btk (Figure 5A).

Semiquantitative RT-PCR for the same set of lineage marker genes of clones 16 and 20 (3 days in IL-7/OP9/1000 ng/mL doxycycline) showed an almost comparable gene expression profile to clone 4 induced to huPax5 expression with low levels of doxycycline (Figure 5B).

Therefore, these genes are prime candidates for the determination and stabilization of the B-lymphoid-myeloid biphenotypic state of cellular development. To further support this hypothesis, we have analyzed FACS-purified biphenotypic cells generated within 4 days from clone 4 (0, 10, and 300 ng/mL doxycycline) as well as from clones 16 and 20 (±1000 ng/mL doxycycline). Semiquantitative RT-PCRs were performed for the set of lineage marker genes that we had previously tested (Figure 5C-D). Interestingly, almost the same RT-PCR expression patterns were observed, as the ones obtained with the unpurified mixture of clone 4 cells induced to huPax5 expression with low levels of doxycycline on IL-7/OP9 (Figure 5C-D).

In conclusion, these findings suggest that concomitant transcription of myeloid- and lymphoid-related genes in Pax5−/− rtTA-huPax5 biphenotypic cells is induced by low levels of huPax5. This expression pattern can, in fact, be stabilized in a cytokine milieu favoring lymphopoiesis as well as myelopoiesis.

**Discussion**

During development of B lymphocytes from hematopoietic stem cells, the transcription factor Pax5 is essential for committing common lymphoid progenitors, the precursors of B-lymphoid, T-lymphoid, NK, and dendritic cells to enter the B-lymphocyte pathway.21 Pax5 exerts a dual role by activating the expression of B cell–specific genes6 and by repressing B-lymphocyte–inappropriate
genes, such as those specific for T lymphocyte–, NK cell–, or myeloid cell–specific functions. Our differential microarray expression and semiquantitative RT-PCR analyses of Pax5+/− pro-pre-B-cell clones and with the same clones induced by doxycycline-dependent, high-level expression of huPax5 confirm these analyses. We add here the differential microarray and semiquantitative RT-PCR analyses of Pax5 that we observe at this level of huPax5 expression both in vitro as expected way of change in expression, a selected group of genes expected way of change in expression, a selected group of genes remain expressed, or are even up-regulated. These genes (Figure 5) are not specific for B-lymphocyte functions, but appear in majority specific for myeloid cell development and function. We take this as indication that they are involved in the development of the B-lymphoid/myeloid B220+/CD11b−/CD19− biphenotypic cells that we observe at this level of huPax5 expression both in vitro as

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Microarray analysis was performed on total RNA extracted from Pax5+/− huPax5 pro-pre-B-cell clone 4, induced to express huPax5 at different levels of doxycycline (0, 10, 300, and 1000 ng/mL) in IL-7/OP9 for 3 days in vitro. Presented are characteristic examples for the differential expression of B-, T-, NK-, and myeloid-lineage–related genes at different levels of doxycycline.

FC indicates fold change; Dox, doxycycline; and rT, retroviral transactivator.
well as in vivo. The capacity of these biphenotypic cells to develop myeloid cells needs to be investigated in greater detail in future experiments. However, it is already evident that they might not be potent as precursors for all myeloid cell types because the microarray and semiquantitative RT-PCR analyses show that granulocyte-macrophage CSF2 receptor keeps being up-regulated, but the macrophage CSF1 receptor is down-regulated.

The gene encoding the transcription factor C/ebpα, one of the genes selectively up-regulated in our biphenotypic cells has been found to be essential for granulocyte development and is expressed in immature myeloid cells. It appears to inhibit monocyte formation, an observation that could be in line with the possibility that not all myeloid lineage cells might develop from our biphenotypic cells. Transduction of C/ebpα, together with Pu.1, has been found to reprogram fibroblasts to macrophage-like cells, again suggesting that the expression of C/ebpα is essential for the development of some of the myeloid cells.

Figure 5. Coexpression of genes characteristic for both lymphoid and myeloid cells in Pax5+/− huPax5 pro-/pre-B-cell clones 4, 16, and 20 before and on induction with low levels of doxycycline in vitro. (A) Pax5+/− huPax5 pro-/pre-B-cell clone 4 was induced to express huPax5 by different levels of doxycycline (0, 10, 300, and 1000 ng/mL) in IL-7/OP9 for 3 days in vitro. Semiquantitative RT-PCR analysis for the expression of lymphoid (Id2, Gata-3, Notch-1, CD19, and Blnk) and myeloid (C/ebpα, CsF2r, Pu.1, C/ebpδ, and CsF1r) related genes was performed on total RNA extracted after 3 days of conditional huPax5 expression at low and high levels of doxycycline. At low levels of doxycycline stimulation (0-300 ng/mL), the expression of lymphoid (Notch-I, Id-2) as well as myeloid (C/ebpα, CsF2r, Pu.1, and CsF1r) related genes can be observed. However, at high levels of doxycycline stimulation (1000 ng/mL) especially, B-lineage–specific genes are expressed and myeloid-related genes are suppressed. (B) Pax5+/− huPax5 pro-/pre-B-cell clones 16 and 20 were induced to express huPax5 by 1000 ng/mL doxycycline in IL-7/OP9 for 3 days in vitro. Gene transcription levels are comparable with clone 4 stimulated with low doxycycline levels. (C-D) Analysis for FACS-sorted B220+/CD19+/CD11b− biphenotypic cells on day 4 of differentiation in Flt3L/SCF/OP9 conditions stimulated with 0, 10, or 300 ng/mL doxycycline (clone 4) or 1000 ng/mL doxycycline (clone 16 and 20). Data are representative of 3 individual experiments.
B-lymphoid/myeloid biphenotypic cells with the capacity to develop to B cells or myeloid cells have been repeatedly detected. Common lymphoid progenitors as well as CD4+/B220+/CD19−/CD93+ progenitors from bone marrow can develop dendritic cells, indicating that this lymphoid cell type can be generated from a progenitor cell capable of pan-lymphoid B-lymphoid development. B220+/CD11b− cells expressing also CD117, CD24, CD43, the Csf1 receptor, Rag-2, TdT and, notably, Pax5 were grown in vitro from bone marrow in the presence of Flt3 ligand. They could be developed to either macrophages or to B cells, depending on the cytokine conditions of the cultures. Not by phenotype, but by function, our biphenotypic cells are reminiscent of those progenitors that are seen early in fetal development, namely bipotential CD93−/B220+/CD19− cells that can differentiate either to B cells or to macrophages. In this report, a CD93+/B220+/CD11b+ cell was also identified where the capacity to develop to B-lymphoid–like or to macrophage-like cells was 1:4. Similar cells could be contained in bone marrow cell populations that have the capacity to develop to B-lymphoid and to myeloid cells that are CD93+/B220+/CD117+/CD19−.

Our findings that low levels of huPax5 expression induce and, in the presence of SCF/Flt3/OP9, stabilize such a lymphoid/myeloid progenitor pose the question of whether biphenotypes observed by others, for example, by ectopic expression of Pax5, might have resulted from low-level Pax5 expression. CLPs, and the Pax5+/− CLP-like progenitors, as well as the Pax5-induced differentiated cells, such as CD19+ pre-B-1 cells and the B220+/CD19+/CD11b− biphenotypic cells express Rag-1 and Rag-2, hence, are genetically unstable, prone to abnormal chromosomal translocations. Therefore, if abnormal levels of Pax5 arrest progenitors at abnormal stages of development, Pax5 may have the effect of an oncogene, as seen in ALLs or acute myeloid leukemias. However, leukemias arising in the earliest stages of embryonic development, different affinities to promoter and enhancer elements of different, critically important genes regulate hematopoietic cell development. Our results with huPax5 are reminiscent of findings by Nutt et al., Dakic et al., and DeKoter and Senger that show differential commitment to myeloid or lymphoid cell differentiation, dependent on the concentration of the transcription factor Pu.1 expressed in hematopoietic progenitor cells.

With our in vitro and in vivo doxycycline-controllable huPax5 expression system in Pax5-deficient pro-/pre-B cells, we have developed a cell-culture system that can, in principle, be adopted to study the in vitro and in vivo effects of any protein or miRNA on central and peripheral B-cell development.

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Authorship

Contribution: S.S. performed most of the experiments, analyzed the data, and wrote the manuscript; M.K., C.D., and I.W. participated in some of the experiments and discussed the outcomes; H.-J.M. performed the microarray analyses; C.B. provided the retroviral expression vectors; and F.M. designed the research, supervised the experiments, and wrote and revised the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for S.S. is Immunology Frontier Research Center, Laboratory of Cellular Dynamics, Osaka University, Osaka, Japan.

Correspondence: Fritz Melchers, Max Planck Institute for Infection Biology, Lymphocyte Development Group, Charitéplatz 1, 10117 Berlin, Germany, e-mail: melchers@mpiib-berlin.mpg.de.
References


Biphenotypic B-lymphoid/myeloid cells expressing low levels of Pax5: potential targets of BAL development

Szandor Simmons, Marko Knoll, Christopher Drewell, Ingrid Wolf, Hans-Joachim Mollenkopf, Corinne Bouquet and Fritz Melchers