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Loss of Pax5 Promotes Plasma Cell Differentiation

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Summary

Pax5 is indispensable for the commitment of early lymphoid progenitors to the B cell lineage as well as for the development of B cells. To better understand the functional importance of Pax5 at the later stages of B cell differentiation, we established a Pax5-deficient DT40 B cell line. The *Pax5*$^{-/-}$ cells exhibited slower growth, decreased surface IgM expression, and total loss of B cell receptor signaling. Moreover, the expression of the plasma cell-characteristic transcription factors Blimp-1 and XBP-1 were significantly upregulated and the expression of Bcl-6 diminished in the *Pax5*$^{-/-}$ cells, and this alteration was normalized by restored Pax5 expression. The Pax5-deficient cells further manifested substantially elevated secretion of IgM into the supernatant, another characteristic of plasma cells. These results indicate that downregulation of Pax5 function promotes the plasma cell differentiation of B cells.

Introduction

The commitment of progenitor cells to the B cell lineage is critically controlled by the transcription factor Pax5 (also known as the B cell-specific activator protein, BSAP). In Pax5-deficient mice, B cell development is arrested at an early pro-B cell stage in the bone marrow (Urbánek et al., 1994), and pro-B cells of *Pax5*$^{-/-}$ mice show characteristics of uncommitted progenitors by retaining a broad lympho-myeloid developmental potential (Nutt et al., 1999; Rolink et al., 1999; Schaniel et al., 2002). This multilineage potential is suppressed by restoration of Pax5 expression, which also rescues the pro-B cell arrest of *Pax5*$^{-/-}$ mice (Nutt et al., 1999). Thus, Pax5 can be considered as a key regulator of the B cell lineage commitment and early B cell development.

Pax5 facilitates the commitment of progenitor cells by activating B cell-specific genes and simultaneously suppressing expression of genes that are inappropriate for the B cell development (reviewed in Busslinger, 2004). Pax5 promotes B cell differentiation by activating the transcription of Igα (CD79a, mb-1) (Fitzsimmons et al., 1996; Nutt et al., 1998), BLNK (SLP-65) (Schebesta et al., 2002), and CD19 (Kozmik et al., 1992; Nutt et al., 1998). These three proteins function in the pre-BCR-signaling pathway, which constitutes a critical developmental checkpoint in the B cell differentiation. Moreover, Pax5 binds to the *VpreB* and $\lambda 5$ promoters (Tian et al., 1997) and participates in the assembly of pre-BCR complex itself, as it also activates expression of the μ chain by controlling the second V_H-DJ_H recombination step of the IgH gene (Nutt et al., 1997; Hesslein et al., 2003). Ectopic Pax5 expression in T lymphocytes induces distal V_H-DJ_H recombination and locus contraction of IgH (Fuxa et al., 2004; Hsu et al., 2004). Furthermore, evidence that Pax5 is essential for sterile κ transcription during Igκ chain gene rearrangement (Tian et al., 1997; Sato et al., 2004) indicates a role for Pax5 also in the regulation of IgL chain rearrangement.

Pax5 is exclusively expressed in the B lineage of lymphoid differentiation from the pro-B to the mature B cell stage (Barberis et al., 1990; Adams et al., 1992), but not in terminally differentiated plasma cells (Barberis et al., 1990). It is believed that B lymphocyte-induced maturation protein 1 (Blimp-1)-dependent repression of Pax5 is required for the plasma cell development (Lin et al., 2002), which is further supported by the fact that enforced Pax5 expression appears to prevent B cell activation-induced plasmacytic differentiation (Usui et al., 1997; Morrison et al., 1998; Lin et al., 2002). Conditional inactivation of Pax5 leads to the total loss of identity and function of mature mouse B cells (Horcher et al., 2001), indicating that Pax5 is required to maintain B cell identity in late B cell development. Moreover, Pax5 binds to the activation-induced cytidine deaminase (AID) promoter, and enforced expression of Pax5 can induce AID expression in pro-B cell lines (Gonda et al., 2003), suggesting that Pax5 may also have an active role in the regulation of AID expression. AID is indispensable for somatic hypermutation (SHM), class switch recombination (CSR), and immunoglobulin gene conversion (Muramatsu et al., 2000; Arakawa et al., 2002), which are central events of B cell development and activation that diversify rearranged *Ig* genes to produce different isotypes of high-affinity antibodies. Hence, Pax5 appears to have an important function also in the late B cell development and activation.

Due to the pro-B cell arrest of *Pax5*$^{-/-}$ mice (Urbánek et al., 1994), the role of Pax5 in later stages of B lineage development is difficult to study in murine system. To gain insight, we established a novel Pax5-deficient DT40 B cell line. The chicken B cell line DT40 is surface IgM positive, but continues Ig gene conversion and seems to be arrested at the bursal B cell stage (Buerstedde et al., 1990). We show here that Pax5$^{-/-}$ DT40 cells exhibited retarded growth, decreased sIgM expression, and total loss of BCR signaling characterized by downregulation of Igβ, BLNK, and Lyn. More importantly, the expression of plasmacytic transcription factors Blimp-1 and X-box binding protein 1 (XBP-1) were significantly

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upregulated in the Pax5-deficient cells with a concomitant decrease of Bcl-6 transcription. *Pax5*$^{-/-}$ cells further manifested their plasmacytic phenotype by showing substantially elevated secretion of IgM into the supernatant. The altered expression levels of Blimp-1, XBP-1, and Bcl-6 were normalized by reintroduction of Pax5 expression. Our results provide evidence that Pax5 not only maintains the identity of B cells but also inhibits plasma cell differentiation.

Results

Disruption of the *Pax5* Gene in DT40 Cells

We disrupted the *Pax5* gene of DT40 cells by using different targeting constructs for both of the alleles (Figure 1A). The DNA binding motif critical for the function of Pax5 is encoded by base pairs 48–429 of the coding sequence. Targeted integration of the *pax5-neo* vector into the third exon of the *Pax5* gene leads to the deletion of coding base pairs 192–405 and the deletion of about 1.5 kb from the third intron sequence. Attempts to generate *Pax5*$^{-/-}$ cells by transfection of a *bsr* construct similar to *pax5-neo* were unsuccessful, since this construct integrated preferentially into the already targeted allele and not into the remaining wild-type allele. Therefore, a different *pax5-bsr* vector was constructed (Figure 1A) whose integration into the third exon of the second *Pax5* allele resulted in the deletion of the coding base pairs 192–306.

The targeted integration of the *pax5-neo* and *pax5-bsr* constructs was verified by genomic PCR by means of the *Pax5*-specific primer 2f with the selection marker-specific primers (neo-f and bsr-f) and a subsequent Southern blot (Figure 1B) with the *Pax5*-specific probe p2 (Figure 1A). Analysis by polymerase chain reaction with reverse transcription confirmed that the *Pax5*$^{-/-}$ DT40 cells did not express full-length Pax5 transcripts (Figure 1C). The absence of Pax5 was further verified by the Pax5-specific antibodies. Moreover, we restored the expression of Pax5 in *Pax5*$^{-/-}$ cells (*Pax5*$^{-/-}$/Pax5 cells; Figure 1D) in order to study the complementation of the phenotype.

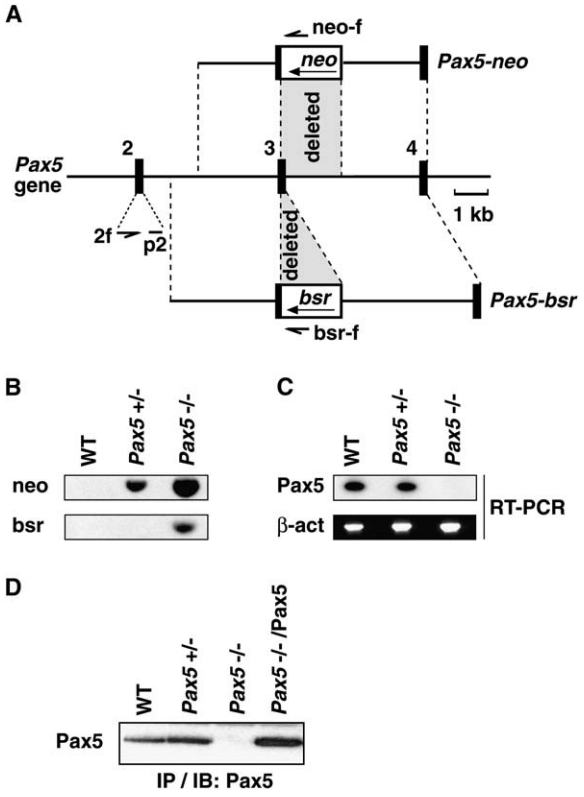

Figure 1. Gene Targeting of the *Pax5* Locus in DT40 B Cells

(A) Schematic presentation of the targeting constructs *Pax5-neo* and *Pax5-bsr* containing neomycin and blasticidin resistance markers, respectively. Coding exons 2–4 of *Pax5* gene are numbered.

(B) Targeted integration in the *Pax5* locus was monitored by genomic PCR reactions in which *Pax5*-specific primer 2f was paired with selection marker-specific primers neo-f or bsr-f. The obtained genomic PCR products were hybridized with the *Pax5*-specific primer p2 to verify the identity of the amplified fragments.

(C) Southern blot of RT-PCR to analyze Pax5 expression of wild-type (wt), *Pax5*$^{+/-}$, and *Pax5*$^{-/-}$ cells.

(D) Immunoprecipitation (IP) of Pax5 followed by immunoblot (IB) with Pax5-specific antibody.

Impaired Cellular Growth and Loss of Functional B Cell Identity in the Absence of Pax5

The Pax5-deficient cells exhibited a slower growth rate and were not able to achieve cell densities comparable to the wild-type cells. Reintroduction of the Pax5 did not fully restore the cellular growth. However, the growth of the *Pax5*$^{-/-}$/Pax5 cells was more comparable to that of wild-type cells (Figure 2A), indicating that the fast proliferation and high cell density characteristic of wild-type DT40 is dependent on Pax5. Surface expression of IgM was clearly decreased, but not entirely abolished, in Pax5-deficient cells. Restoration of Pax5 expression reconstituted sIgM expression (Figure 2B), demonstrating a role for Pax5 in the maintenance of normal BCR expression level at the cell surface. In contrast, cell surface expression of chB6, which is expressed in all avian B cells (Pink and Rijnbeek, 1983), was unaltered in the absence of Pax5.

To investigate the role of Pax5 in BCR signaling, we analyzed the calcium influx induced by sIgM crosslinking. In the absence of Pax5, calcium influx induced by BCR signaling was totally abolished (Figure 3A). To further study the nature of the BCR-signaling defect, we analyzed the phosphorylation of cytoplasmic signaling molecules after BCR crosslinking or stimulation of the cells with H_2O_2, which bypasses the antigen-driven BCR signal. Phosphorylation induced by BCR crosslinking was totally abolished in the *Pax5*$^{-/-}$ cells (Figure 3B), but H_2O_2-induced phosphorylation still occurred. However, the pattern of phosphorylated proteins in the Pax5-deficient cells was quite different compared to the wild-type cells (Figure 3C). These findings indicate that there is a major defect or even total block in early steps of BCR signaling, which can be overcome in part if the initial events of BCR signaling are bypassed with H_2O_2.

To define the BCR-signaling defect in the Pax5-deficient cells more precisely, we compared the gene-expression profiles of the *Pax5*$^{-/-}$ and wild-type DT40 cells by using a novel BursaEST array (ArrayExpress accession: A-MEXP-155). The array contains about 15,000 probes in duplicate that are derived from the bursal B cells. Array analysis (ArrayExpress accession: E-MEXP-270) revealed downregulation of Igβ, BLNK, and Lyn related to the wild-type levels (Table 1). The results

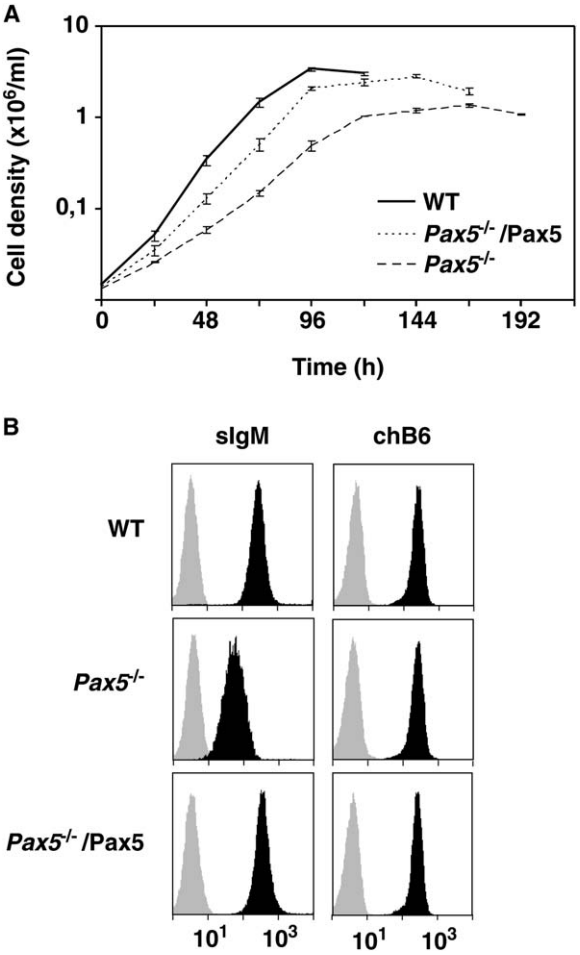

Figure 2. Cell Growth and Surface Expression in the Absence of Pax5

(A) Time versus cell density growth curves of wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells, showing slower growth in DT40 *Pax5* mutants. Error bars indicate mean SD of independent experiments (n = 3).

(B) Flow cytometric analysis of surface immunoglobulin (sIgM) and chB6 expression as indicated by logarithmic fluorescence intensity versus relative cell number.

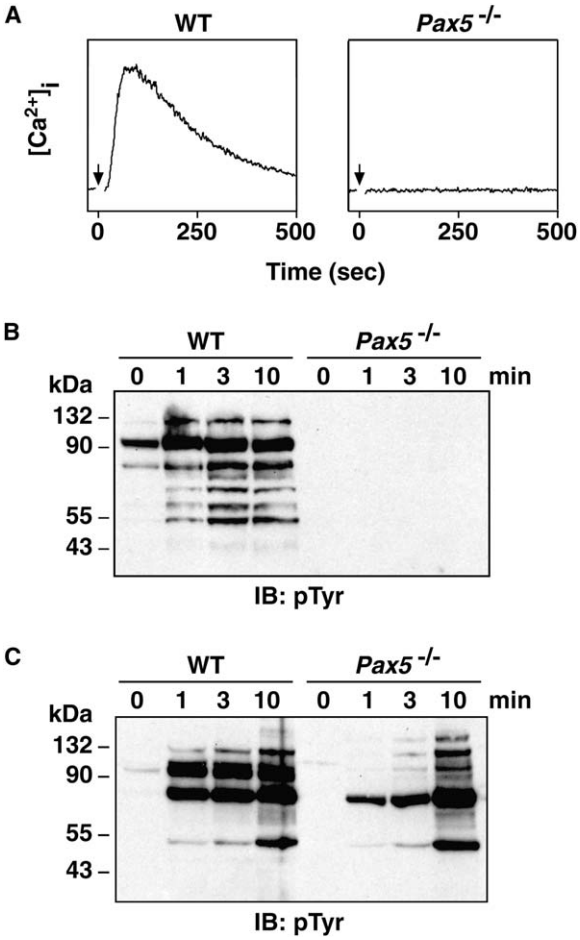

Figure 3. BCR Signaling in Pax5-Deficient Cells

(A) Comparison of calcium mobilization in wild-type (wt) and *Pax5*$^{-/-}$ cells following the BCR crosslinking, at the time indicated by the arrow.

(B) Phosphorylation of cytoplasmic signaling molecules following BCR crosslinking. Phosphotyrosine immunoblots of whole-cell lysates were prepared before BCR engagement and 1, 3, or 10 minutes after BCR engagement.

(C) Phosphorylation of cytoplasmic signaling molecules following stimulation with H_2O_2.

on these BCR-signaling genes were confirmed by real-time quantitative PCR (Q-PCR), which showed that the expression levels of Igβ, BLNK, and Lyn were decreased in the Pax5-deficient cells to 6%, 39%, and 16% of wild-type levels, respectively. Expression of Igβ, BLNK, and Lyn were restored to wild-type levels in the *Pax5*$^{-/-}$/Pax5 back transfectant cells. Moreover, further quantitative PCR analysis showed that the expression level of Btk in the *Pax5*$^{-/-}$ cells was about 50% of the expression level in the wild-type cells (Table 1). Thus, Pax5-deficient DT40 cells have lost their functional B cell identity, as they express only low levels of surface IgM and fail to respond to sIgM crosslinking, and the expression levels of several critical components of the BCR-signaling machinery are downregulated.

Loss of Pax5 Induces IgM Secretion and Plasmacytic Differentiation

Interestingly, the array analysis and quantitative PCR revealed that the expression of VpreB3 was significantly downregulated with a concomitant increase in Ig λ light chain (IgL) mRNA expression in the Pax5$^{-/-}$ DT40 cells (Table 1). The downregulation of VpreB3 expression was indeed due to the loss of Pax5, as Pax5 reexpression in the *Pax5*$^{-/-}$/Pax5 cells restored VpreB3 expression. VpreB3 is expressed at high level from the pro-B to the immature B cell stage, where it binds to free IgL chains, preventing their maturation and secretion (Rosnet et al., 2004). Hence, the diminished VpreB3 expression could imply increased secretory activity in the *Pax5*$^{-/-}$ cells.

Pax5 is also known to repress the expression of XBP-1 (Reimold et al., 1996), IgH (Singh and Birshtein, 1993), and J chain (Rinkenberger et al., 1996; Wallin et al., 1998, 1999), which are involved in the Ig secretion. Considering this and given the diminished VpreB3 expression in the absence of Pax5 (Table 1), we next analyzed the IgM secretion and the expression levels of transcription factors XBP-1, Blimp-1, and Bcl-6, which are differentially expressed during the plasma cell differentiation.

Table 1. Differentially Expressed Genes in Wild-Type, *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 Cells

	Pax5$^{-/-}$		*Pax5*$^{-/-}$/Pax5
Differentially Expressed Genes	Array	Q-PCR	Q-PCR
CD79b/Igβ	0.4	0.06	1.07
BLNK	0.53	0.39	1.38
Lyn	0.33	0.16	0.94
Btk		0.5	n.d.
VpreB3	0.18	0.03	0.89
IgL		2.55	n.d.
HSP70	5.8	9.7	n.d.
ITM2A	0.53	0.38	n.d.
HMG-17	0.45	0.45	n.d.
Caspase 3	0.53	0.43	n.d.

The expression levels obtained by BursaEST array analysis or by quantitative PCR (Q-PCR) are given as values compared to the expression level in wild-type cells, which is given the value 1. The results are expressed as fold differences. n.d., not done.

According to quantitative PCR analysis, expression of the plasmacytic transcription factors XBP-1 and Blimp-1 were clearly upregulated in the absence of Pax5 (Figure 4A). Restoration of Pax5 expression in the *Pax5*$^{-/-}$/Pax5 cells normalized Blimp-1 transcription to the level observed in the wild-type cells. The expression of XBP-1 was decreased significantly in the *Pax5*$^{-/-}$/Pax5 cells compared to the Pax5-deficient cells, but it remained slightly upregulated compared to the wild-type cells. These findings suggest that Pax5 is needed to suppress both XBP-1 and Blimp-1, which are essential for the plasmacytic differentiation. The number of Bcl-6 transcripts diminished in the *Pax5*$^{-/-}$ cells compared to the wild-type cells (Figure 4A). In the *Pax5*$^{-/-}$/Pax5 cells, in which Pax5 is overexpressed (Figure 1D), Bcl-6 expression was restored and even upregulated compared to the wild-type cells (Figure 4A), indicating the involvement of Pax5 in the maintenance of Bcl-6 transcripts. Collectively, the upregulation of XBP-1 and Blimp-1 with diminished Bcl-6 expression are indications for early plasmacytic differentiation of *Pax5*$^{-/-}$ cells.

XBP-1 promotes Ig secretion by coordinating various cellular changes that are needed for the secretory phenotype of plasma cells (Shaffer et al., 2004). Given that XBP-1 mRNA is spliced by IRE1 in response to stress in the endoplasmic reticulum (ER) and that only the spliced form of XBP-1 can activate the unfolded protein response (UPR) and secretion (Yoshida et al., 2001), we next analyzed the XBP-1 splice variants. The wild-type cells expressed both isoforms of XBP-1 at low level, whereas in the *Pax5*$^{-/-}$ and *Pax5*$^{-/-}$/Pax5 cells, the spliced form of XBP-1 was predominantly expressed at the mRNA level (Figure 4B), indicating that XBP-1 is spliced after the Pax5 deficiency. However, in contrast to XBP-1 expression, apparently Pax5 does not control XBP-1 splicing, as it remains unaltered regardless of restored Pax5 expression.

We next analyzed the IgM secretion of Pax5-deficient DT40 cells. The relative number of transcripts coding the secretory form of IgM heavy chain (μS) was increased more than 10-fold in the *Pax5*$^{-/-}$ cells compared to the wild-type cells (Figure 4C). In contrast, the expression level of the membrane form of IgM heavy chain (μM) was not altered. Reintroduction of Pax5 expression decreased the relative number of μS transcripts in the *Pax5*$^{-/-}$/Pax5 cells, although not back to the level of wild-type cells. The ELISPOT analysis of the *Pax5*$^{-/-}$ cells indicated elevated frequency of cells secreting IgM, which was decreased by the restored Pax5 expression (Figure 4D). Moreover, the pulse chase metabolic labelling with L-[^{35}S]methionine and L-[^{35}S]cysteine followed by IgM immunoprecipitation clearly showed that more IgM was secreted into the supernatant of the *Pax5*$^{-/-}$ cells compared to the wild-type cells. The IgM secretion was reduced, although not to the level of wild-type cells, by reintroduction of Pax5 expression (Figure 4E), which is in line with the observation that XBP-1 is spliced in the *Pax5*$^{-/-}$/Pax5 cells (Figure 4B).

Pax5 Is Needed to Maintain an Adequate Level of Bcl-6

Considering that Bcl-6 is needed to suppress the Blimp-1 expression (Reljic et al., 2000; Shaffer et al., 2000; Tunyaplin et al., 2004) while the Blimp-1 expression contributes to the inhibition of Bcl-6 expression (Shaffer et al., 2002), we next addressed the question of whether downregulation of Bcl-6 or upregulation of Blimp-1 would constitute the primary effect in the absence of Pax5.

To test whether Bcl-6 is sufficient to suppress the Blimp-1 expression in the absence of Pax5, we transfected Bcl-6 into the *Pax5*$^{-/-}$ DT40 cells. In the independent transfectant clones (Bc1, Bc2, and Bc3), Blimp-1 expression was downregulated close to the level observed in the wild-type cells (Figure 5A), thus indicating that Pax5 is not required for the inhibition of Blimp-1. However, enforced Bcl-6 expression had no effect on the upregulated XBP-1 expression of the Pax5$^{-/-}$ cells (data not shown), verifying that Pax5, not Bcl-6, is required to suppress XBP-1. Furthermore, we wanted to test whether the downregulation of Pax5 is necessary for the suppression of Bcl-6 by Blimp-1. To study this, we transfected Blimp-1 into the *Pax5*$^{-/-}$/Pax5 cells having an inactive endogenous *Pax5* locus and ectopically enforced Pax5 expression. In the transfectant cells (Px/B1), Blimp-1 was able to downregulate Bcl-6 expression regardless of the ectopic Pax5 expression (Figure 5B), indicating that Blimp-1 does not inhibit the Bcl-6 expression via suppression of Pax5 expression. However, enforced Blimp-1 expression did not alter the downregulation of XBP-1 expression in the Px/B1 cells (data not shown), suggesting that Blimp-1 upregulates XBP-1 expression via suppression of Pax5 expression.

In summary, these results provide evidence that Pax5 is not needed to inhibit Blimp-1 expression, suggesting that a drop in the level of Bcl-6 is likely to cause the Blimp-1 induction in the absence of Pax5, and indicating that Pax5 is needed for the maintenance of Bcl-6 rather than for the inhibition of Blimp-1.

Pax5 Regulates the Expression of AID and the Transcription Factors Aiolos and EBF in DT40 Cells

Given that the putative regulatory region of *AID* contains binding sites for Pax5 and that AID transcription can be induced by enforced Pax5 expression in pro-B cell lines (Gonda et al., 2003), we next examined the transcription of AID in the *Pax5*$^{-/-}$ DT40 cells. The relative number of

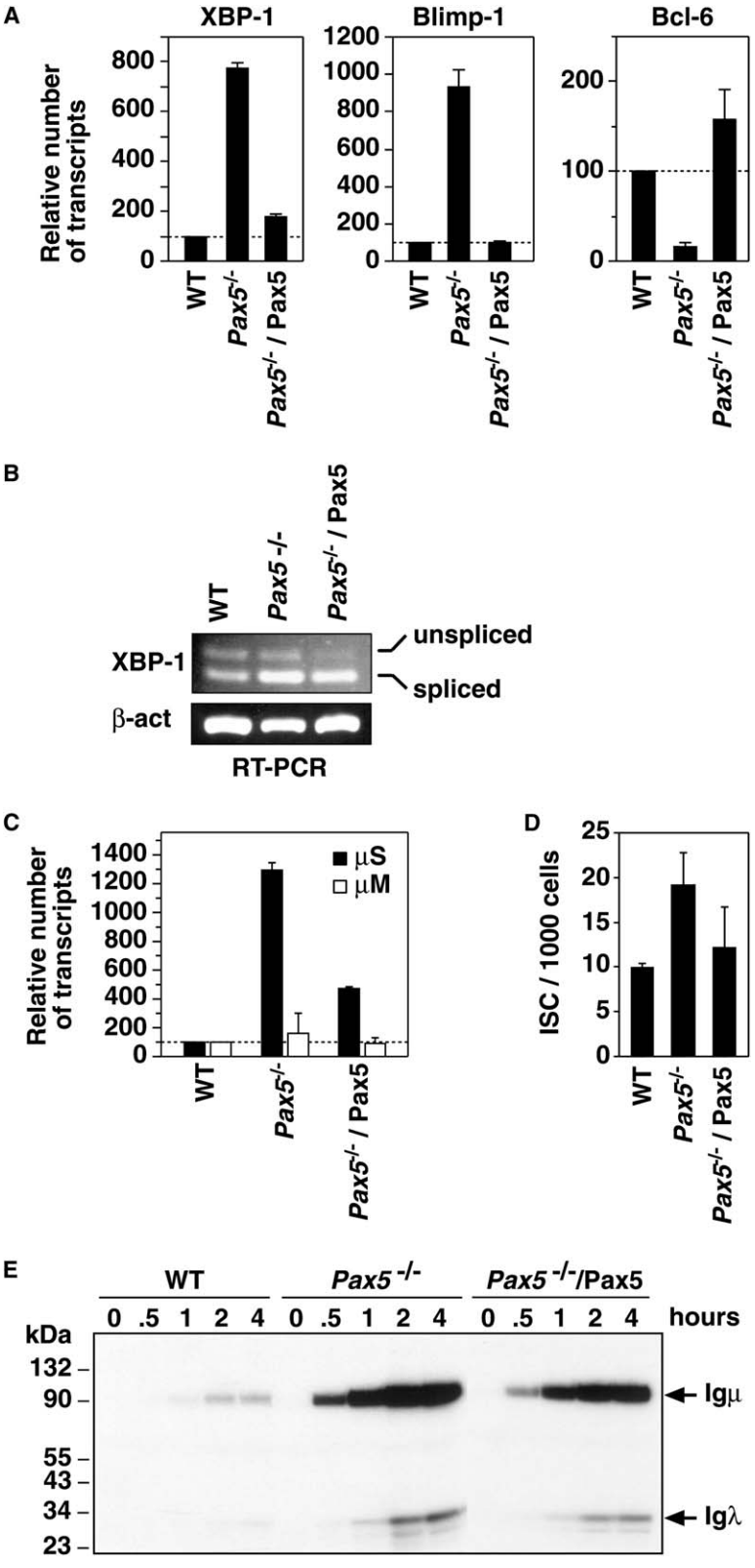

Figure 4. Analysis of Pax5-Deficient DT40 Cells for Signs of Plasmacytic Differentiation

(A) Analysis of XBP-1, Blimp-1, and Bcl-6 expression in wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells by quantitative PCR. Mean SD of four independent experiments (n = 4) is indicated by error bars. The relative number of transcripts in the *Pax5*$^{-/-}$ and *Pax5*$^{-/-}$/Pax5 cells is shown in comparison to the level of transcripts in the wild-type cells, which is given the value 100 and indicated by a dashed line.

(B) RT-PCR analysis of XBP-1 splice variants in wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells.

(C) Quantitative PCR analysis of the secretory (μS) and the membrane (μM) form of μ heavy chain transcripts. Error bars indicate mean SD of independent experiments (n = 4). The relative number of transcripts in the *Pax5*$^{-/-}$ and *Pax5*$^{-/-}$/Pax5 cells is shown as a comparison to the level of transcripts in the wild-type (wt) cells, which is given the value 100 and indicated by a dashed line.

(D) Enzyme-linked immunospot (ELISPOT) assay of wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells. Error bars indicate mean SD of independent experiments (n = 3) for each sample.

(E) Pulse-chase metabolic labelling followed by IgM immunoprecipitation for wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells. Secreted IgM was immunoprecipitated from the cell culture supernatant after 0, 30 min, 1 hr, 2 hr, and 4 hr chase times. Components of the secreted IgM (Igμ and Igλ) are indicated with arrows.

AID transcripts in the Pax5-deficient cells was diminished by 98% compared to the wild-type cells (Figure 5C). However, in the *Pax5*$^{-/-}$/Pax5 cells overexpressing the Pax5 (Figure 1D), the relative number of AID transcripts was more than 4-fold greater than in the wild-type cells (Figure 5C), showing that Pax5 positively regulates the AID expression in B cells. Furthermore, cells from clone Bc2 expressing Bcl-6 in the absence of Pax5 had abolished AID expression, in contrast to the Px/B1 cells expressing both Blimp-1 and

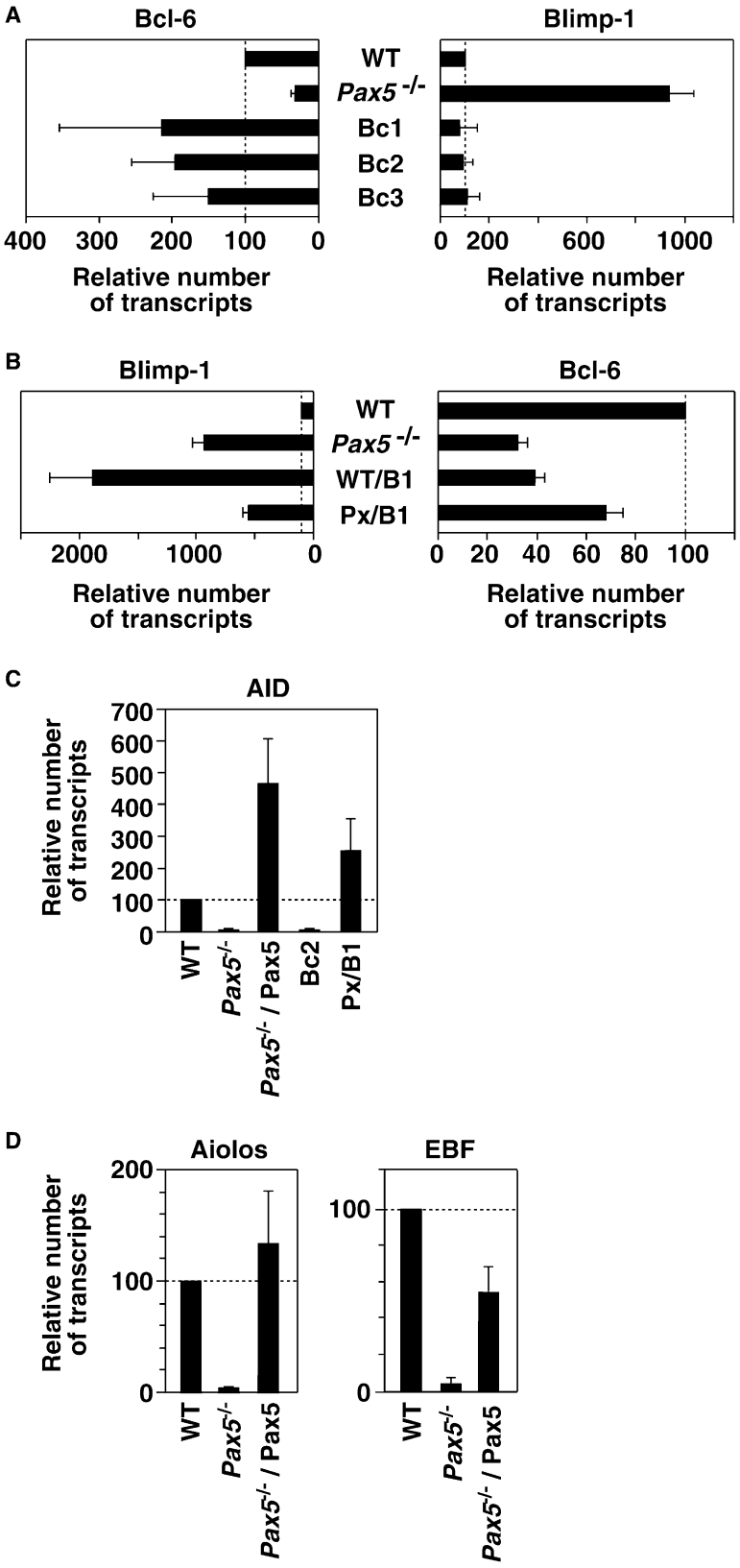

Figure 5. Quantitative PCR Analysis of Bcl-6 and Blimp-1 Transfectants and Influence of Pax5 on the Expression of AID, Aiolos, and EBF

(A) Comparison of Bcl-6 and Blimp-1 transcripts in wild-type (wt), *Pax5*$^{-/-}$, and Bcl-6 transfectant Pax5-deficient cells (clones Bc1, Bc2, and Bc3) by quantitative PCR. The mean SD of three independent experiments (n = 3) is indicated by error bars.

(B) Comparison of Blimp-1 and Bcl-6 transcripts in wild-type (wt), *Pax5*$^{-/-}$, Blimp-1 transfectant wild-type (WT/B1), and Blimp-1 transfectant Pax5$^{-/-}$/Pax5 (Px/B1) cells by quantitative PCR. The mean SD of three independent experiments (n = 3) is indicated by error bars.

(C) Quantitative PCR analysis of the AID transcripts in wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells, as well as in the clone Bc2 (expressing Bcl-6 in the absence of Pax5) and in the Px/B1 cells (expressing both Pax5 and Blimp-1 ectopically). The mean SD of four independent experiments (n = 4) is indicated by error bars.

(D) Analysis of the Aiolos and EBF expression in wild-type (wt), *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells by quantitative PCR. The mean SD of four independent experiments (n = 4) is indicated by error bars.

In all panels the relative number of transcripts is shown as a comparison to the level of transcripts in the wild-type cells, which is given the value 100 and indicated by a dashed line.

Pax5, which had comparable AID expression to *Pax5*$^{-/-}$/Pax5 cells (Figure 5C). Thus, the expression of AID is definitely dependent on Pax5.

We also analyzed the expression of transcription factors, which may have importance in B cell fate decisions. While the expression of Ikaros, which is indispensable

for the B cell development, was unaltered in the absence of Pax5 (data not shown), the relative number of Aiolos and EBF transcripts were diminished in the *Pax5*$^{-/-}$ cells (Figure 5D). The reintroduction of Pax5 restored the Aiolos expression and increased the relative number of EBF transcripts, suggesting that Pax5 is needed for the sustained expression of both Aiolos and EBF.

Discussion

Here we show that loss of Pax5 function promotes plasma cell differentiation, as our *Pax5*$^{-/-}$ DT40 cells exhibit several features of plasmacytic development. In plasma cell differentiation, the expression of Blimp-1 and XBP-1 is upregulated with simultaneous downregulation of Bcl-6 (reviewed in Calame et al., 2003), which is exactly what we observed in our *Pax5*$^{-/-}$ cells (Figure 4A). Moreover, XBP-1 has a major role in the induction of Ig secretion (Reimold et al., 2001; Shaffer et al., 2004), and IgM secretion was clearly increased in our Pax5-deficient DT40 cells (Figure 4E). Plasma cells are also characterized by the loss of functional B cell identity (Calame et al., 2003), as the membrane BCR is low or absent and increased Blimp-1 expression downregulates the transcription factors, which are required for effective BCR signaling (Shaffer et al., 2002). In line with this, our Pax5-deficient B cells had decreased sIgM expression (Figure 2B) and abolished BCR signaling (Figures 3A and 3B), most likely caused by the downregulation of several molecules involved in the BCR-signaling pathway (Table 1). Increased Blimp-1 expression was associated with impaired growth in our *Pax5*$^{-/-}$ cells (Figures 2A and 4A), in accordance with the observation that multiple factors promoting proliferation and growth are inhibited by Blimp-1 in plasmacytic cells (Lin et al., 1997; Shaffer et al., 2002). Taken together, the impaired growth, decreased sIgM expression, loss of BCR signaling, increased expression of Blimp-1 and XBP-1, and induction of IgM secretion in Pax5-deficient cells are clear indications of plasmacytic differentiation. Therefore, our results demonstrate that loss of Pax5 function is sufficient to promote plasma cell differentiation.

Our findings that Pax5 deficiency causes both downregulation of Bcl-6 and upregulation of Blimp-1 (Figure 4A) leads to an intriguing question about which one of the events is the primary consequence of *Pax5* targeting. Enforced Blimp-1 expression has been shown to drive plasma cell differentiation (Turner et al., 1994; Schliephake and Schimpl, 1996; Piskurich et al., 2000). It has also been demonstrated that Bcl-6 expression is elevated in the germinal centers, where it prevents the premature induction of Blimp-1 (Shaffer et al., 2000; Tunyaplin et al., 2004). However, once the plasmacytic differentiation starts, Blimp-1 is thought to directly repress the Pax5 expression (Lin et al., 2002) and to contribute to the downregulation of Bcl-6 (Shaffer et al., 2002). Suppression of both Pax5 and Bcl-6 is critical for the plasma cell development, as enforced expression of either Bcl-6 or Pax5 inhibits plasmacytic differentiation (Usui et al., 1997; Morrison et al., 1998; Reljic et al., 2000; Lin et al., 2002), and Pax5 also represses the expression of XBP-1 (Reimold et al., 1996), which is needed for the plasma cell formation and Ig secretion (Reimold et al., 2001; Shaffer et al., 2004). The fact that Pax5 deficiency causes increased Blimp-1 expression and decreased Bcl-6 expression (Figure 4A) suggests that Pax5 is needed either to inhibit Blimp-1 expression or to sustain adequate Bcl-6 levels. However, our results indicate that Pax5 is not needed for the suppression of Blimp-1, as enforced Bcl-6 expression can downregulate Blimp-1 in the *Pax5*$^{-/-}$ cells (Figure 5A). Moreover, transfected Blimp-1 can downregulate Bcl-6 in the *Pax5*$^{-/-}$/Pax5 DT40 cells (Figure 5B), possibly due to the double-negative regulatory circuit involving the Bcl-6 and Blimp-1 proteins postulated by previous studies (Shaffer et al., 2002; Tunyaplin et al., 2004; Fujita et al., 2004). Therefore, Pax5 appears to be needed for the maintenance of Bcl-6, leading to the conclusion that decrease of Bcl-6 level is likely to constitute the primary event in the induction of plasmacytic phenotype in the *Pax5*$^{-/-}$ cells (Figure 6).

The conditional inactivation of *Pax5* in mice results in the loss of B cell identity (Horcher et al., 2001) and similarities to our Pax5-deficient B cell model including the reduced level of surface BCR as well as the downregulation of BLNK, EBF, and Igβ (B29) expression. However, the conditional inactivation of *Pax5* in mice by a CD19-cre does not lead to the increased Blimp-1 expression nor Ig secretion (Horcher et al., 2001) observed in our model system. This discrepancy may be related to the differences in B cell development pathways between the mouse and avian species. However, we believe that a more likely explanation could be a consequence of the c-*myc* overexpression in DT40 cells (Neiman et al., 2001). c-*myc* is a key regulator of cell proliferation expressed in strongly dividing but not in resting quiescent cells. Moreover, proliferation has an essential role in the B cell activation and terminal differentiation where c-*myc* is downregulated by Blimp-1 (Lin et al., 1997). Given that enforced Blimp-1 expression has been reported to induce apoptosis in the immature or only partially activated B cells (Messika et al., 1998) and that our results reveal a spontaneous Blimp-1 expression without activation or proliferation in the *Pax5*$^{-/-}$ DT40 cells, it remains possible that in vivo Pax5-deficient mature B cells die through apoptosis as a result of the Blimp-1 activation and the consequent c-*myc* downregulation. Furthermore, conditional gene targeting of *Pax5* via the CD19-cre (Horcher et al., 2001) inactivates *Pax5* already at an early stage of B cell development and may therefore result in the reversion of the B cell commitment rather than terminal differentiation, as suggested by studies with conditionally *Pax5*-inactivated pro-B cells (Mikkola et al., 2002). However, our findings in *Pax5*$^{-/-}$ DT40 cells strongly indicate that Pax5 is needed to inhibit plasma cell differentiation at later stages of B cell development. In line with our results are findings that low Pax5 expression in B cells from multiple myeloma patients is associated with increased Blimp-1 expression (Borson et al., 2002).

Pax5 was suspected to positively regulate AID expression, as it is known to bind to the AID promoter and enforced expression of Pax5 can induce AID expression (Gonda et al., 2003). Our results unequivocally show that AID expression is dependent on Pax5 function, consistent with the observation that the expression of AID is inhibited during plasmacytic differentiation. Introduction of Blimp-1 expression into B cells has been shown to promote downregulation of AID (Shaffer et al., 2002).

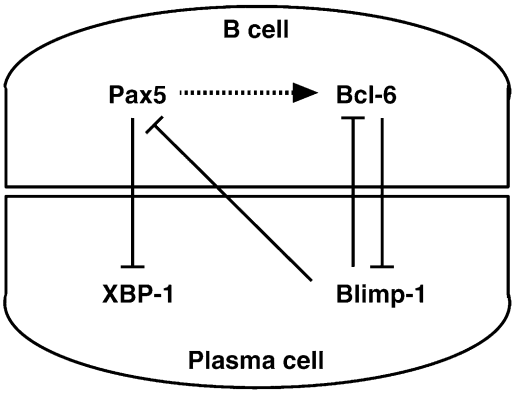

Figure 6. A Schematic Diagram of Regulatory Network Controlling Plasma Cell Differentiation

Our results suggest the diminishing of Bcl-6 as a primary event in the development of plasmacytic phenotype in Pax5-deficient DT40 cells, since Pax5 is not essential to the inhibition of Blimp-1. Thus, Pax5 is more likely needed to maintain Bcl-6 level (dashed arrow) at later stages of B cell development. The solid lines indicate the previously known inhibitory signals of this regulatory network.

However, our findings that enforced Blimp-1 expression in the *Pax5*$^{-/-}$/Pax5 cells did not inhibit the expression of AID (Figure 5C) indicate that Blimp-1 expression leads to the downregulation of AID during plasma cell differentiation via suppression of Pax5 expression rather than by inhibiting AID directly. Thus, Pax5 likely contributes to the regulation of SHM, CSR, and Ig gene conversion by maintaining AID expression. In accordance, mice carrying conditionally inactivated *Pax5* alleles are characterized by reduced IgG class switching (Horcher et al., 2001), which could be due to diminished AID expression.

Our data show that Pax5 inhibits the plasmacytic development, because the loss of Pax5 function is sufficient to promote the Ig secretion and plasma cell differentiation. We suggest that the biological significance of Pax5 expression in mature B cells is to first allow sufficient clonal expansion accompanied by AID-mediated CSR and Ig gene diversification. Once B cells with high affinity for antigen are selected from this pool, Pax5 is downregulated and promotes their terminal differentiation into plasma cells. Recently, Pax3 was shown to function in melanocyte differentiation as a single factor responsible for cell fate determination and prevention of terminal differentiation (Lang et al., 2005). Based on our results, we propose that Pax5 has similar function in lymphocyte development promoting B cell commitment and inhibiting plasma cell differentiation. Thus, fate determination coupled to inhibition of terminal differentiation may be a general aspect of the Pax family of transcriptional regulators.

Experimental Procedures

Cells and Antibodies

Wild-type and *Pax5* mutant DT40 cells were maintained in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 50 μM β-mercaptoethanol, 2 mM L-glutamine, penicillin, and streptomycin. Cells were cultured at 40ºC with 5% CO_2. Anti-chB6 mAb (L22) was described previously (Pink and Rijnbeek, 1983). Anti-Pax5 mAb (A-11) and anti-pTyr mAb (PY99) were purchased from Santa Cruz Biotechnology. Anti-chicken IgM mAbs (M4 and M1) and anti-chicken λ light chain mAb (L1) were purchased from Southern Biotechnology Associates Inc.

Targeting Vectors and Generation of the Pax5-Deficient DT40 B Cell Line

In the gene-targeting construct *Pax5-neo*, the selection cassette was flanked by 2.0 and 2.5 kb of the chicken *Pax5* sequence in the 5′ and 3′ sides, respectively (Figure 1A). The 5′ flanking arm was obtained by PCR from genomic DNA of DT40 cells by means of the primers 2f 5′-GTGAACCAGCTGGGGGGCGTTTTTGTGAAT-3′ and 3Lr 5′-TTTAGATCTGATCATCCAATCACTCCAGGCTAAATGCTCC-3′. The 3Lr primer added *BglII* and *BclI* sites to the genomic PCR-product. An internal *BamHI* site within the *Pax5* intron 2 was used together with the *BglII* site (created by 3Lr) to clone the fragment into pUC18 vector. The 3′ flanking arm was obtained by genomic PCR by using primers 3Rf 5′-ATTGCAGAGTACAAACGCCAAAATCCCACCA-3′ and 4Rr 5′-TTTGGATCCGGCTGCTGCACCTTTGTCCGTATGAT-3′. The 4Rr primer introduced a *BamHI* site, which was used for cloning together with the internal *BclI* site within the *Pax5* intron 3. The 3′ flanking arm was cloned the into the *BclI* site (created by 3Lr) of the 5′ flanking arm that had been cloned into pUC18 vector. The neomycin resistance marker was cloned into the *BclI* site between the two flanking *Pax5* sequences.

In the *Pax5-bsr* construct, the selection cassette was flanked by 3.0 and 4.0 kb of the *Pax5* sequence on the 5′ and 3′ sides, respectively (Figure 1A). The 5′ arm of the *Pax5-bsr* was obtained by genomic PCR with primers b2Lf 5′-CCGCGTCGACCACGGTACCGTCAGCTAAATACTCGGCA-3′and b3Lr 5′-TGGTGTCGACCCTCCAATCACTCCAGGCTAAATGCTC-3′, both creating *SalI* sites, which were used in cloning of the PCR product into MCS I of the pLoxBsr vector (Arakawa et al., 2001). The 3′ flanking arm of the *Pax5-bsr* was obtained by genomic PCR with primers b3Rf 5′-AAAATTGCAGAGTACTAGTGCCAAAATCCCACCA-3′ and b4Rr 5′-TTGGGCGGCCGCTGCACCTTTGTCCGTATGAT-3′. The b3Rf created a *SpeI* and the b4Rr a *NotI* site, which were used to clone the 3′ arm into MCS II of the pLoxBsr vector.

The *Pax5-neo* and *Pax5-bsr* were linearized by *BamHI* and *Acc65I* digestion, respectively, and introduced into DT40 cells by electroporation at 710 V, 25 μF. Stable transfectant clones were selected in the presence of 2 mg/ml G418 (*Pax5-neo* transfectants) or 50 μg/ml blasticidin S (*Pax5-bsr* transfectants). *Pax5*-targeted clones were identified based on two genomic PCR reactions (Figure 1B), in which the *Pax5*-specific primer 2f was used with the selection cassette-specific primer neo-f 5′-GCGCATCGCCTTCTATCGCCTTCTTGACGAG-3′ or bsr-f 5′-CGATTGAAGAACTCATTCCACTCAAATATACCC-3′. The obtained PCR-products were hybridized with the *Pax5*-specific probe 2p 5′-GTCAGCCACGGCTGCGTCAGCAAAATACT-3′.

RT-PCR Analysis

Pax5 expression of wild-type DT40, *Pax5*$^{+/-}$, and *Pax5*$^{-/-}$ clones was analyzed by RT-PCR. The cDNA from 1 × 10^5 cell equivalent, which was prepared as indicated in Nera et al. (2006), was amplified with primers Pax5-f 5′-GTCAGCCACGGCTGCGTCAGCAAAATAC-3′ and Pax5-r 5′-GGCTGCTGCACCTTTGTCCGTATGAT-3′. A PCR reaction with the chicken β-*actin*-specific primers b1-f 5′-GTGCTGTGTTCCCATCTATCGT-3′ and b1-r 5′-TGGACAATGGAGGGTCCGGATT-3′ was used as a positive control. Southern hybridization was performed with the *Pax5*-specific primer Pax5-p 5′-ATTAAGCCTGGAGTGATTGGAGGATCAA-3′.

Expression Vectors and Transfections

The coding sequence of chicken Pax5, Bcl-6, and Blimp-1 was amplified from DT40 cDNA with the primers Px5-Hf 5′-TATAAGCTTCGCAATGGATTTGGAGAAGATGTA-3′ and Px5-Br 5′-TATAGATCTGCTTTGGGTCCGAGGTCAGTG-3′ (for Pax-5), Bc6-Hf 5′-AAAAAGCTTATGGCCTCACCGGCAGACAGCTGCA-3′ and Bc6-Nr 5′-AAAGCTAGCTCAGCAAGCCTTGGGGAGCTCCGGA-3′ (for Bcl-6), and B1-Nhf 5′-AAAGCTAGCATGAAAATGGACATGGAGGATGCT-3′ and B1-Ncr 5′-AAACCATGGTTAAGGGTCCATTGGTTCAACTGT-3′ (for Blimp-1). PCR products were cloned into the pExpress vector (Arakawa et al., 2001) and the insert was sequenced. The expression cassettes containing the cloned PCR products between the chicken β-actin promoter and the SV40 poly-A sequence were excised from pExpress as *SpeI* cassettes, which were subsequently cloned into

pLoxPuro (Pax5 and Bcl-6) or pLoxHisD (Blimp-1) vectors (Arakawa et al., 2001). The vectors were linearized and transfected to the *Pax5*$^{-/-}$ (Pax5 and Bcl-6), wild-type, or *Pax5*$^{-/-}$/Pax5 (Blimp-1) cells at 710 V, 25 μF. Stable transfectants were selected in the presence of 0.5 μg/ml puromycin (Pax5 and Bcl-6) or 1 mg/ml histidinol (Blimp-1), and the expression of transfected gene was verified by immunoblots (Pax5) or RT-PCR (Bcl-6 and Blimp-1).

Immunoprecipitation and Western Blot Analysis

Lysates from 5 × 10^6 wild-type, *Pax5*$^{+/-}$, *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells were prepared as described previously (Nera et al., 2006) and sequentially incubated with anti-Pax5 mAb (A-11) and protein A-agarose at 4ºC overnight. The immunoprecipitates were washed four times with the lysis buffer, and the samples were denatured at 75ºC for 10 min in the SDS sample buffer.

In Western blot analysis, whole-cell lysates or immunoprecipitates were separated by 4%–12% SDS-PAGE, transferred onto nitrocellulose membranes, and detected by appropriate antibodies and the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech). Phosphotyrosine immunoblots for whole-cell lysates (1 × 10^6 cells/sample) were performed as described previously (Nera et al., 2006).

Analysis of Cellular Growth

Wild-type, *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cell cultures were diluted to 10^4 cells/ml and samples were harvested at 24 hr intervals. The cell densities were analyzed by flow cytometer using TruCOUNT tubes (Becton Dickinson) according to the manufacturer's instructions.

Calcium Measurements

Cells (10^6) were suspended in buffered solution containing 20 mM HEPES, 5 mM glucose, 1 mM $CaCl_2$, 0.25 g/l BSA, and 0.25 mM sulfinpyrazone (Sigma) in PBS (pH 7.4) and were loaded with 3 μM Fluo-3 AM (Molecular Probes) for 45 min at room temperature. Following the loading period, cells were washed three times and incubated an additional 30 min to ensure the complete cleavage of acetoxymethyl group from Fluo-3. Cells were washed twice, and continuous monitoring of fluorescence from cell suspension (1 × 10^6/ml) was performed at 37ºC using a FacsCalibur flow cytometer at the excitation wavelength of 488 nm and an emission wavelength of 530 nm. Cells were stimulated with 4 μg/ml of M4 mAb. The average signal curve was measured by calculating the average of the fluorescence value of the events at every time point.

BursaEST Array Analysis

The BursaEST array (ArrayExpress accession: A-MEXP-155, at http://www.ebi.ac.uk/arrayexpress) contains 14,592 clones on a nylon filter macroarray. The mRNA was isolated from wild-type DT40 and *Pax5*$^{-/-}$ cells, hybridized, and visualized with a phosphoimage-reader (Fuji). A reasonable false discovery rate was chosen (about 5%) for the Significance Analysis of Microarrays (SAM) method to define differential expression. Detailed methods and data are available at the ArrayExpress (EBI, Hinxton, UK) under the accession E-MEXP-270 and in the Supplemental Data available with this article online.

Quantitative Real-Time PCR

Messenger RNA was isolated from cell lines by the mRNA Isolation Kit (Roche) and used as a template to create cDNA by the 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche). The quantitative real-time PCR analysis was made using LightCycler equipment (Roche) and the SYBR Green detection method. The LightCycler analysis was made using LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to manufacturers' instructions. 2 μl of serial template dilutions were used. The Mg^{2+} concentration and PCR conditions were optimized for each primer pair. Melting curve detection was run after each analysis. GAPDH, β-actin, and elongation factor 1 (EF-1) were used to normalize the cDNA concentration. Primer sequences are provided in the Supplemental Data.

ELISPOT Assay

The number of immunoglobulin secreting cells (ISC) was enumerated by the enzyme-linked immunospot (ELISPOT) assay. Microtiter plates were coated with antibody to chicken IgM (M1). Nonspecific binding sites were blocked with 1% bovine serum albumin in PBS. Cell suspensions were incubated in the wells for 3 hr. A biotin-labeled anti-chicken IgL antibody (L1) was added and the plates were incubated overnight at room temperature. Streptavidin-conjugated alkaline phosphatase (Prozyme) were added and incubated for 2 hr in 37ºC. The substrate was added in hot 3% agarose, and ISC were enumerated by counting the spots in the wells under a light microscope.

Pulse-Chase Metabolic Labeling and IgM Precipitation

Wild-type, *Pax5*$^{-/-}$, and *Pax5*$^{-/-}$/Pax5 cells (4 × 10^7 of each) were washed twice with PBS at room temperature. Each cell sample was suspended to 1 ml of the methionine and cysteine-free DMEM medium (GIBCO) supplemented with 10% dialyzed FCS and was incubated 30 min at 40ºC. Next, 200 μCi/ml of the Redivue PROMIX L-[^{35}S] in vitro cell labeling mix (Amersham Biosciences) was added and the cells were incubated for 15 min at 40ºC. Following this, 4 ml of normal DMEM medium (GIBCO) containing 10% FCS and an excess (5 mM) of L-cysteine (Sigma) and L-methionine (Sigma) was added, and each sample was divided into five independent 1 ml sample cultures (each containing 8 × 10^6 cells/ml), which were incubated for the indicated chase times (0, 30 min, 1 hr, 2 hr, and 4 hr). After incubation, the carefully cleared supernatants from the samples were subjected to the IgM immunoprecipitation.

For immunoprecipitation, 50 μg of anti-IgM mAb (M1) were conjugated to 500 μl of protein A/G-PLUS-agarose reagent (Santa Cruz Biotechnology) for 12 hr at 4ºC in 1 ml of lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodiumdeoxycholate, 1% SDS, 1 mM EDTA, 2 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, and 1× protease inhibitor "cocktail" [Roche]) and then saturated for 2 hr in PBS containing 5% BSA. After two washes in lysis buffer, the conjugated M1 antibodies were suspended to 500 μl of the lysis buffer. 20 μl of this conjugated M1 suspension was added to each cleared supernatant sample in immunoprecipitation and incubated for 12 hr at 4ºC. The immunoprecipitates were washed four times with the lysis buffer, and the samples were denatured at 75ºC for 10 min in SDS sample buffer and separated by 4%–12% SDS-PAGE. The radioactive gels were fixed in 15% methanol, 7.5% acetic acid and treated with Enlightning autoradiography enhancer (PerkinElmer) before drying and exposure on autoradiographic film.

Supplemental Data

Supplemental Experimental Procedures can be found with this article online at http://www.immunity.com/cgi/content/full/24/3/283/DC1/.

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