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Letter to the Editor

Re: Torlakovic *et al*. PU.1 protein expression has a positive linear association with protein expression of germinal centre B cell genes including *BCL-6*, *CD10*, *CD20* and *CD22*: identification of PU.1 putative binding sites in the *BCL-6* promotor. *J Pathol* 2005;206:312–319

In a recent issue of *The Journal of Pathology*, Torlakovic *et al* reported the expression analysis of BCL6 and Pu.1 proteins in a large number of follicular lymphomas and diffuse large B-cell lymphomas (DLBCLs) [1]. A strong, positive association of these two protein markers was observed in these germinal centre (GC)-derived tumours. A previous study by the same group had detected high-level Pu.1 expression in normal GC B cells which coincided with the expression pattern of BCL6 [2], a transcription repressor and master regulator of the GC programme. In their *Journal of Pathology* article, the authors also identified three putative Pu.1 binding sites in the *BCL6* promoter region that could be recognized by Pu.1 in gel shift assays. Based on this information and the co-expression pattern, Torlakovic *et al* suggested that Pu.1 is a positive regulator of BCL6. They further hypothesized that Pu.1 may play an important role in GC B-cell maturation, possibly through regulating BCL6 and other B-cell genes such as CD20 and GANP. We read this article with great interest due to our own on-going studies of BCL6 expression regulation. As shown in Figure 1, the results from our functional analysis argue against a positive role for Pu.1 in regulating the endogenous *BCL6* gene in mature B cells.

We first tested the effect of Pu.1 on *BCL6* transcription in reporter assays using transient transfection. In the Mutu III cell line, which expresses neither BCL6 nor Pu.1, transcription from a 6 kb *BCL6* reporter was inhibited by BCL6 in a dose-dependent manner due to the negative autoregulation mechanism [3]. In comparison, Pu.1 did not show any notable effect on this reporter when transfected at a low dose (0.1 pmol); at a higher dose (0.3 pmol), however, Pu.1 increased the reporter activity by three-fold, an effect that was observed at all BCL6 dosages and thus independently of BCL6 autoregulation (Figure 1A). This result does offer some support to the proposal by Torlakovic *et al*, but because reporter assays do not fully recapitulate the chromatin environment where transcription regulation of endogenous genes takes place, we next analysed the response of the endogenous *BCL6* gene to fluctuations in Pu.1 levels. Ly10 is an activated B-cell-type diffuse large B-cell lymphoma (ABC-DLBCL) cell line that has low levels of endogenous BCL6 but no Pu.1 (Figure 1B). When transfected with a Pu.1

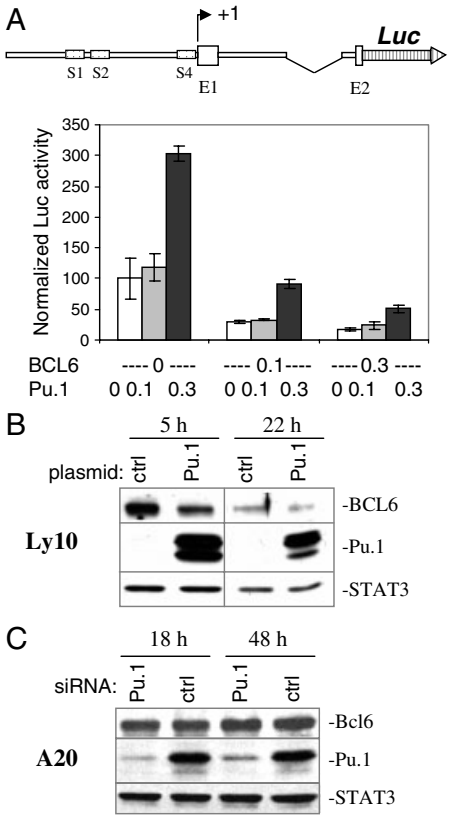

Figure 1. The role of Pu.1 in BCL6 expression was investigated in three types of functional assay. (A) In *BCL6* reporter assays, a positive role for Pu.1 was only observed when a high dose of Pu.1 plasmid was used. Mutu III cells were transfected by using the superfect reagent with the 6 kb human *BCL6* reporter construct, pLA/s5wt, and the indicated amounts of BCL6 and Pu.1 expression plasmids. All transfections were performed in duplicate and included a β-gal reporter plasmid used to control transfection efficiency. Cells were harvested 48 h later for luciferase assays. Luciferase counts were normalized to the basal level, which was defined as 100 in the graph. (B) Forced expression of Pu.1 in the ABC-DLBCL cell line Ly10 suppressed endogenous BCL6. Ten million Ly10 cells were transiently transfected with 10 μg of Pu.1 expression plasmids using Nucleofector Kit T. Whole cell lysates were harvested at the indicated time points and analysed for Bcl6 and Pu.1 proteins by western blot. (C) In A20 cells, endogenous BCL6 was insensitive to siRNA-mediated Pu.1 knockdown. Ten million cells were transfected with 20 μg of the Pu.1 siRNA oligo (5′-GGAGGUGUCUGAUGGAGAA-3′) or a control oligo (5′-UUCUCCGAACGUGUCACGU-3′, designed by Qiagen) using Nucleofector Kit T. Whole cell lysates were harvested at the indicated time points for western blot analysis of Bcl6 and Pu.1. In both panel B and panel C, total STAT3 protein is shown as loading control

expression plasmid, Ly10 cells quickly built up notable amounts of Pu.1; however, this change only led to a decrease, rather than an increase, in the endogenous BCL6 levels (Figure 1B). Similar results were obtained from another ABC-DLBCL cell line, Ly3 (not shown). It is known that Pu.1 can activate as well as repress transcription depending on the promoter and cellular context. We reasoned that the observed inhibitory effect of Pu.1 on BCL6 could be due to lack of appropriate Pu.1 co-activator(s) in Ly3 and Ly10 cells. To address this issue, we turned to the siRNA-mediated knockdown approach. A20 is a mouse B-cell lymphoma cell line that expresses high levels of both Bcl6 and Pu.1. As shown in Figure 1C, siRNA treatment substantially reduced the endogenous Pu.1 levels and this knockdown effect was maintained until at least 48 h. During the entire course of the experiment, however, we did not detect any obvious changes in the endogenous Bcl6. We do not believe that the use of a mouse B cell line is an issue here because the mouse *Bcl6* gene has Pu.1-like sites at positions corresponding to sites S1, S2, and S4 identified by Torlakovic *et al* in the human *BCL6* promoter region. These mouse Pu.1-like sites can also be recognized by the Pu.1 protein in gel shift assays (not shown). Collectively, our results in Figures 1B and 1C do not support a positive role for Pu.1 in BCL6 expression. In line with our mechanistic studies is the lack of obvious GC phenotype in mice carrying B-cell-specific Pu.1 deletion, as described in a recent report [4]. During T-cell-dependent antibody responses, these mice formed normal-sized GCs and produced antigen-specific antibodies with the proper titre and affinity [4]. Since Bcl6 is essential for GC formation and function, it is very unlikely that the level of Bcl6 in these mice was notably reduced in the absence of Pu.1.

Our results highlight the importance of studying gene expression regulation using *in vivo* assays and the endogenous locus. It is worth mentioning here that Pu.1 is not the only example of a gene that is highly expressed in GC B cells but has a negligible role in GC function. Expression of HGAL is not only GC-specific, but also has significant prognostic value in DLBCL. Nevertheless, gene knockout studies in mice revealed that the murine homologue of HGAL, M17, is dispensable for normal GC responses [5]. Thus, developmental stage-specific expression patterns do not always translate to essential functions at the corresponding developmental stages.

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