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LETTER TO THE EDITOR

Long-term deregulated human hematopoiesis in goats transplanted *in utero* with *BCR-ABL*-transduced lin$^-$CD34$^+$ cord blood cells

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Dear Editor,

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the oncogenic *BCR-ABL* fusion gene with increased ABL tyrosine kinase (TK) activity [1]. Transduction of primitive hematopoietic cells with the BCR-ABL oncoprotein creates cells that display many features of their *BCR-ABL*$^+$ counterparts in CML patients. Inhibition of the TK activity of BCR-ABL by small molecule inhibitors (imatinib mesylate (IM), dasatinib and nilotinib) [2] causes impressive responses in chronic phase CML patients. Nevertheless, response failures, early relapses and the later emergence of IM-resistant disease remain significant problems for many patients. In particular, CML stem cells are insensitive to IM and other ABL inhibitors and are not eradicated by currently available agents [1, 3]. These findings underscore the importance of understanding the biology of CML stem/progenitor cells and their unique properties *in vivo*, in guiding the development of strategies to permanently cure CML.

We have used a new xenograft model that exploits the advantages of a long-living, large animal host, the goat, which can be transplanted in a preimmune state *in utero* and then followed for several years after birth [4]. We transplanted fetal goats *in utero* with *BCR-ABL*-transduced lin$^-$CD34$^+$ human cord blood (CB) cells and analyzed 6 liveborn goats for persistent engraftment of GFP$^+$*BCR-ABL*$^+$ cells in multiple tissues and for initiation of early phase CML. Twenty-eight fetuses were injected intraperitoneally at 45-55 days of gestation with 2×10^4 to 10^5 transduced lin$^-$ human CB cells (~85% were CD34$^+$, 20%-30% were GFP$^+$*BCR-ABL*$^+$). Another 14 fetuses were similarly transplanted with control GFP-vector-transduced cells (MIG). Both groups displayed a high rate of abortion (> 50%). Therefore, only 6 goats transplanted with *BCR-ABL*-transduced cells (all recipients of 2×10^4 cells, hereafter referred to as BCR-ABL goats for brevity) and 5 goats transplanted with MIG-transduced cells (2-5 $\times 10^4$ cells, hereafter referred to as MIG goats for brevity) were born alive and thus available for follow-up studies.

A group of 3 BCR-ABL goats and 2 MIG goats were sacrificed 3 weeks after birth. Fluorescence microscopy and confocal laser scanning microscopy revealed a large number of GFP$^+$ (*BCR-ABL*$^+$) cells in liver, kidney and lung from all three BCR-ABL goats (Supplementary information, Figure S1A-S1C). FACS analysis also detected GFP$^+$ (*BCR-ABL*$^+$) cells in suspensions prepared from multiple tissues including liver, kidney, smooth muscle, heart and lung of all three BCR-ABL goats (1%-49% of all the viable cells from these tissues, Supplementary information, Figure S2). These results were confirmed by fluorescence *in situ* hybridization (FISH) analyses performed on cells isolated from the bone marrow (BM) or liver of a BCR-ABL goat using the P17H8 probe that specifically identifies the unique α-satellite DNA sequences on human chromosome 17, and a specific probe for human *BCR-ABL* (Figure 1A and Supplementary information, Figure S3A). Neither of these probes gave any signal in the control BM cells from a normal goat (unmanipulated) (Figure 1A). Immunohistochemical staining of liver tissue sections obtained from all three of the 3-week-old BCR-ABL goats showed frequent coincidence of GFP fluorescence and the expression of human proliferating cell nuclear antigen (PCNA) (Supplementary information, Figure S1D).

Interestingly, analyses of the remaining goats at various time points up to 3 years post-transplantation showed that after 6 months, the white blood cell (WBC) count in the peripheral blood (PB) of all three BCR-ABL goats was 3- to 5-fold higher than that in the 3 MIG goats (up to 2.5×10^{10}/l vs 5-10 $\times 10^9$/l, $P < 0.01$, Figure 1C) and in 3 normal goats (data not shown). This difference was sustained for another 2.5 years in all three goats monitored (Figure 1C). GFP$^+$ (*BCR-ABL*$^+$) cells were present in the PB and BM of all BCR-ABL goats (Figures 1B and Supplementary information, Figure S4A). These

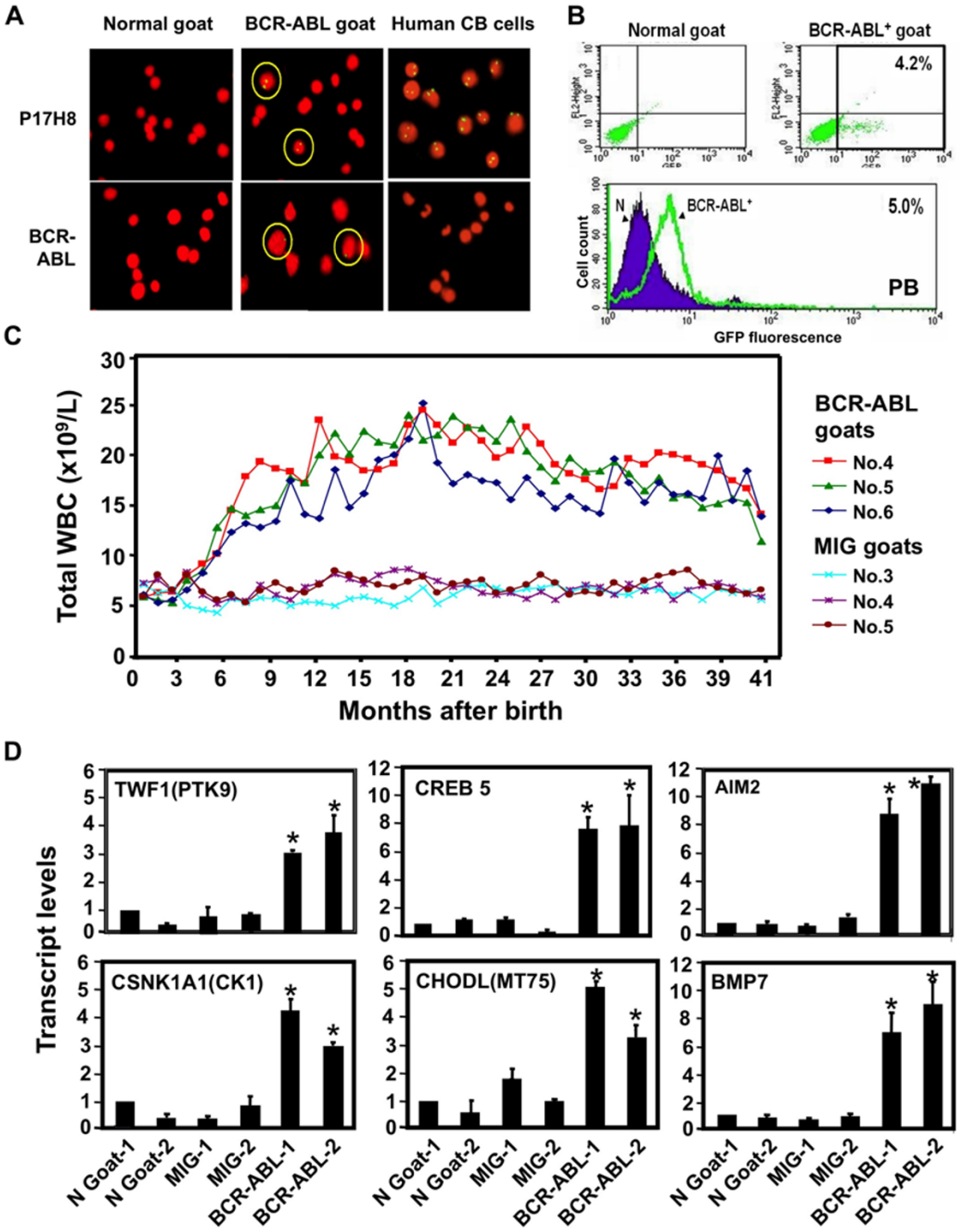

Figure 1 WBC counts and detection of the differential expression of selected human genes in recipient goats. **(A)** Representative FISH data for cells isolated from BM of a normal (unmanipulated) goat and a BCR-ABL goat and data for unmanipulated human CB cells. P17H8 probe was used to detect human α-satellite DNA sequences on chromosome 17 and a specific probe was used to detect human *BCR-ABL*. Positive cells from the BM of a BCR-ABL goat are circled. **(B)** Representative FACS plots (top) and a histogram (bottom) demonstrating the presence of GFP^+ cells in the PB of a BCR-ABL goat compared to a normal goat. **(C)** Total WBC counts measured over a 3-year period in 3 BCR-ABL goats and 3 MIG goats. **(D)** Gene expression levels assessed by RT-qPCR and normalized to *GAPDH* mRNA levels are plotted for selected genes. Expression differences of the indicated genes were measured in PB (top) and liver (bottom) samples from 2 normal goats (N), 2 MIG goats, and 2 BCR-ABL goats. Values shown are the mean ± SEM of triplicate measurements. * Significant difference between BCR-ABL goats and normal or MIG goats ($P < 0.05$).

included human $CD34^+$ (primitive) cells (1%-5% of all BM cells), human $CD14^+$ and $CD15^+$ (maturing myeloid) cells (0.2%-6% and 0.4%-2%, respectively, of all BM cells), human glycophorin A^+ (erythroid, GPA) cells (2%-10%), human $CD20^+$ (B lineage) cells (> 4%) and human $CD7^+$ T lineage cells (0.4%-0.6%). The presence of *BCR-ABL*$^+$ cells in the PB was confirmed by FACS (Figure 1B) and FISH (using the P17H8 and *BCR-ABL* probes, data not shown).

Quantitative PCR (qPCR) analysis of DNA isolated from tissues of BCR-ABL goats (after 3 weeks) detected up to 8×10^4 copies of the *GFP* (*BCR-ABL*) transgene per μg of DNA, with the highest copy numbers in spleen (Supplementary information, Figure S4A). After 10 months, $5\text{-}7 \times 10^4$ copies of the transgene were detected in DNA from BM samples (Supplementary information, Figure S4A). Human *CD34* and *GPA* sequences were also detected in the BM of BCR-ABL goats (up to 10^4 copies of human GPA) or MIG goats (200 copies) but not in normal goats (Supplementary information, Figure S4B). Microarray RNA profiling, as previously validated for chimeric goats [4], demonstrated the existence of viable human cells in MIG and BCR-ABL goats and detected gene expression differences between BCR-ABL, MIG and normal goats (Supplementary information, Tables S1-S6). Differentially-expressed genes in the PB and liver of normal, MIG and BCR-ABL goats, which are relevant to CML due to their roles in cell cycle control or kinase activities, are listed in Supplementary information, Table S7. Several candidate genes from the list (3 from PB and 3 from liver) were tested by reverse transcription-qPCR (RT-qPCR) in the same goat samples assayed by microarrays. The results confirmed the significant gene expression differences in PB and liver samples between BCR-ABL and MIG goats (Figure 1D).

We describe for the first time the *de novo* generation of a human CML model in a large animal recipient of human lin^-CD34^+ CB cells transduced with a retroviral vector encoding a human *BCR-ABL* cDNA. Goats that received relatively small numbers of human *BCR-ABL*-transduced CB cells *in utero* exhibited rapid and long-term deregulated output of hematopoietic cells, including excessive production of $CD34^+$, myeloid and lymphoid cell types. Previous studies have indicated that lin^-CD34^+ cells represent a subset of the normal $CD34^+CD38^-$ hematopoietic stem cell population that display long-term repopulating ability in immunodeficient mice [5] and likely share many properties with cells that sustain the chronic phase of CML in patients. Our large animal xenograft model thus offers the advantages of longevity and cell expansion, which are not possible with other approaches (e.g., transplantation of NOD/SCID mice).

Interestingly, the frequency of *BCR-ABL*$^+$ myeloid cells present in the BM of BCR-ABL goats with elevated WBC counts was not significantly higher than that in the BM of MIG goats with normal WBC counts (Figure 1 and Supplementary information, Figure S4). This indicates that the observed deregulation of hematopoiesis, although consistent among individual animals and sustained for a long period, was largely restricted to the most terminal stages of granulopoiesis. In addition, we found that erythroid cell production was enhanced in BCR-ABL goats (Supplementary information, Figure S4), which parallels previous observations in immunodeficient mice transplanted with *BCR-ABL*-transduced cells [6]. This finding is consistent with the increased production of erythroid progenitors observed in many samples of chronic phase CML patients [7], although an increase in terminal differentiation of erythroblasts in patients is rare. The level of BCR-ABL phosphorylation in *BCR-ABL*-transduced human CB cells was equivalent to that observed in K562 cells [6], a cell line developed from a CML patient with blast phase disease [8]. Thus, some of the features obtained in both mouse and goat *in vivo* xenograft models are more reminiscent of accelerated phase CML, where higher levels of *BCR-ABL* expression are typically detected [3] and perturbations of differentiation are usually more exaggerated. However, from the analyses performed here, there was no indication of a progressive disease process, such as accelerating expansion of the leukemic clone, appearance of splenomegaly or hepatomegaly, or weight loss [6, 9]. The indolent behavior of the leukemic population regenerated in the goat model thus appears to more closely resemble the early chronic phase of CML in humans. An interesting observation was the prevalence of transduced human cells in multiple organs of the engrafted goats, regardless of whether MIG- or *BCR-ABL*-transduced cells had been initially transplanted (Supplementary information, Figure S2). This phenomenon was particularly pronounced in the goats analyzed 3 weeks after birth. Nevertheless, the present data do not definitively establish the basis of the GFP fluorescence observed in the non-hematopoietic tissues analyzed, which awaits future investigations.

Transcript profiling identified thousands of human genes expressed in the PB or liver of the chimeric goats (Supplementary information, Tables S1 and S2), indicating the presence of viable human cells. Only about half of the expressed genes were detected in both tissues, likely reflecting a different distribution of cell types in the two tissues. These genes can be divided into several functional categories, including kinases and other proteins known to regulate cell proliferation, homing, differentiation, transcription, nucleotide binding and ion

transport. The transcript levels of some genes in *BCR-ABL* chimeric tissues were greater than those observed in normal human CB cells (confirmed by RT-qPCR), suggesting elevated gene activity on a per cell basis in the *BCR-ABL*$^+$ cells generated in the goats. Several upregulated genes in the *BCR-ABL*$^+$ cells produced in chimeric goats (e.g., *CREB5*, *CK1*, *TWF1*, *MAD2L1*, and the splicing factor *SRSF1*), were also identified as being upregulated in CML stem/progenitor cells by microarray profiling and serial analysis of gene expression (Supplementary information, Tables S1 and S3, Y Zhao and C Eaves, unpublished results). Upregulated expression of the *WT1* gene identified here is of interest, given the recent report that *WT1* gene expression may correlate with imatinib resistance in patients [10]. Further studies of enriched populations of engrafted *BCR-ABL*$^+$ cells using FACS or laser capture microdissection should provide more detailed molecular profiling data, thereby improving the application of this model in the evaluation of new therapeutics and investigation of mechanisms of disease progression.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)