To the editor:

CD34+ cell dose and the occurrence of GVHD in the presence of in vivo T-cell depletion

Perez-Simon et al have recently reported an association between the development of extensive chronic graft-versus-host disease (cGVHD) and the dose of $CD34^+$ cells infused, in patients undergoing allogeneic transplantation with reduced intensity conditioning.[1] Furthermore, the increase in cGVHD seen with higher $CD34^+$ cell doses is associated with lower disease relapse rates, and the authors conclude that the $CD34^+$ content of the graft should be manipulated to prevent cGVHD in patients at low risk of relapse and to maximize disease control in those who are at high risk. This association is independent of the dose of $CD3^+$ cells infused, and was described in a cohort of 86 patients with HLA-matched sibling donors receiving peripheral blood stem cells (PBSCs) mobilized by granulocyte colony-stimulating factor (G-CSF). This is in keeping with the previously observed correlation between higher $CD34^+$ cell doses and the development of cGVHD in patients receiving unmanipulated PBSCs.[2]

We present 63 patients who have undergone transplantation with reduced intensity conditioning in this institution from June 1997 to June 2003, using fludarabine (30 mg/m^2) from day −7 (D −7) to D −3, melphalan (140 mg/m^2) D −2, and alemtuzumab (either 20 mg/d from D −8 to D −4, or 30 mg/d D −8 and D −7). Of the 63 patients, 50 had HLA-matched sibling donors, and 13 had unrelated donors, of whom 6 were mismatched at up to 2 HLA class I or class II alleles. All the patients received G-CSF–mobilized PBSCs. Cyclosporin (3 mg/kg) was given intravenously from D −1 as prophylaxis for GVHD, and the GVHD occurrence described below relates to the period prior to any subsequent donor lymphocyte infusions (minimum 6 months after transplantation).

The median $CD34^+$ dose was 5.3×10^6/kg (range 0.9-21.1 $\times$ 10^6/kg). Rates of grades II-IV acute GVHD and chronic GVHD were 12 (19%) of 63 and 12 (20%) of 59, respectively, for the entire patient group, with extensive chronic GVHD occurring in 9 (15%) of 59 patients. For sibling donors, the acute GVHD and cGVHD rates were 14% and 17% (extensive chronic GVHD, 13%), and for the unrelated donors, 38% and 33% (extensive chronic GVHD, 25%), respectively. This compares with 30% grades II-IV acute GVHD and 56% cGVHD (extensive cGVHD, 31%) in the sibling cohort reported by Perez-Simon et al.[1]

In our experience, no significant association could be observed between the occurrence of acute or chronic GVHD (limited or extensive) and the $CD34^+$ dose infused for the cohort as a whole (Figure 1), or when subanalyzed by donor type, although this must be interpreted with caution as the numbers affected are small. Furthermore, no impact on event-free or overall survival could be demonstrated with increasing

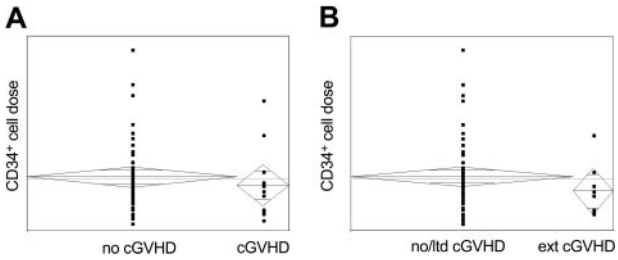

Figure 1. Correlation between CD34+ cell dose and the occurrence of chronic GVHD. All chronic GVHD, both limited and extensive. (B) Extensive chronic GVHD.

$CD34^+$ cell dose, and similarly, no impact of $CD34^+$ cell dose on the timing of onset or persistence of chronic GVHD was seen.

In addition, despite similar $CD34^+$ doses, the occurrences of both acute and particularly chronic GVHD were markedly less than in the paper of Perez-Simon et al.[1] This is presumed to reflect the effect of in vivo alemtuzumab, both in depleting T cells from the incoming graft and potentially in depleting antigen-presenting cells from the recipient.[3-5]

We would thus conclude that, in reduced intensity transplantation with in vivo T-cell depletion, increasing $CD34^+$ dose is not associated with increased incidence of extensive chronic GVHD, and that manipulation of the $CD34^+$ content of the graft may not result in therapeutic benefit.

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To the editor:

Promoter hypermethylation of the cyclin-dependent kinase inhibitor (CDKI) gene *p21WAF1/CIP1/SDI1* is rare in various lymphomas and carcinomas

The cyclin-dependent kinase inhibitor (CDKI) p21 can act as a tumor suppressor to inhibit tumor cell growth.[1] In contrast to other CDKI genes, *p21* is rarely mutated or deleted in tumors.[1] Alternative mechanisms of *p21* inactivation have been suspected, with *p21* hypermethylation demonstrated in some hematologic and solid tumors.[1-9] Therefore, we read with great interest the 2 contrasting

reports on *p21* methylation in *Blood*,[2,3] and we would like to share our results of *p21* methylation.

CpG islands (CGIs) are frequently silenced by methylation in tumors.[10] The *p21* promoter and exon 1 are within a typical CGI (Figure 1A). We examined its expression and methylation in 46 tumor cell lines (6 Hodgkin disease [HD], 1 leukemia, 33 carcinomas, 6 Burkitt lymphoma [BL]) and 12 normal peripheral blood mononuclear cell (PBMC) samples. *p21* was readily expressed in all the samples, except silenced in Rael (Figure 1B). Methylation analysis of *p21* in 58 cell lines, 12 normal PBMCs, and 10 normal tissues, using methylation-specific polymerase chain reaction (MSP),[10] showed that this promoter was consistently unmethylated in all the samples (Table 1), except it was weakly methylated in Raji and strongly methylated in Rael.

We further examined *p21* methylation in 187 primary tumors (lymphomas and carcinomas). Only 3 lymphomas showed methylation

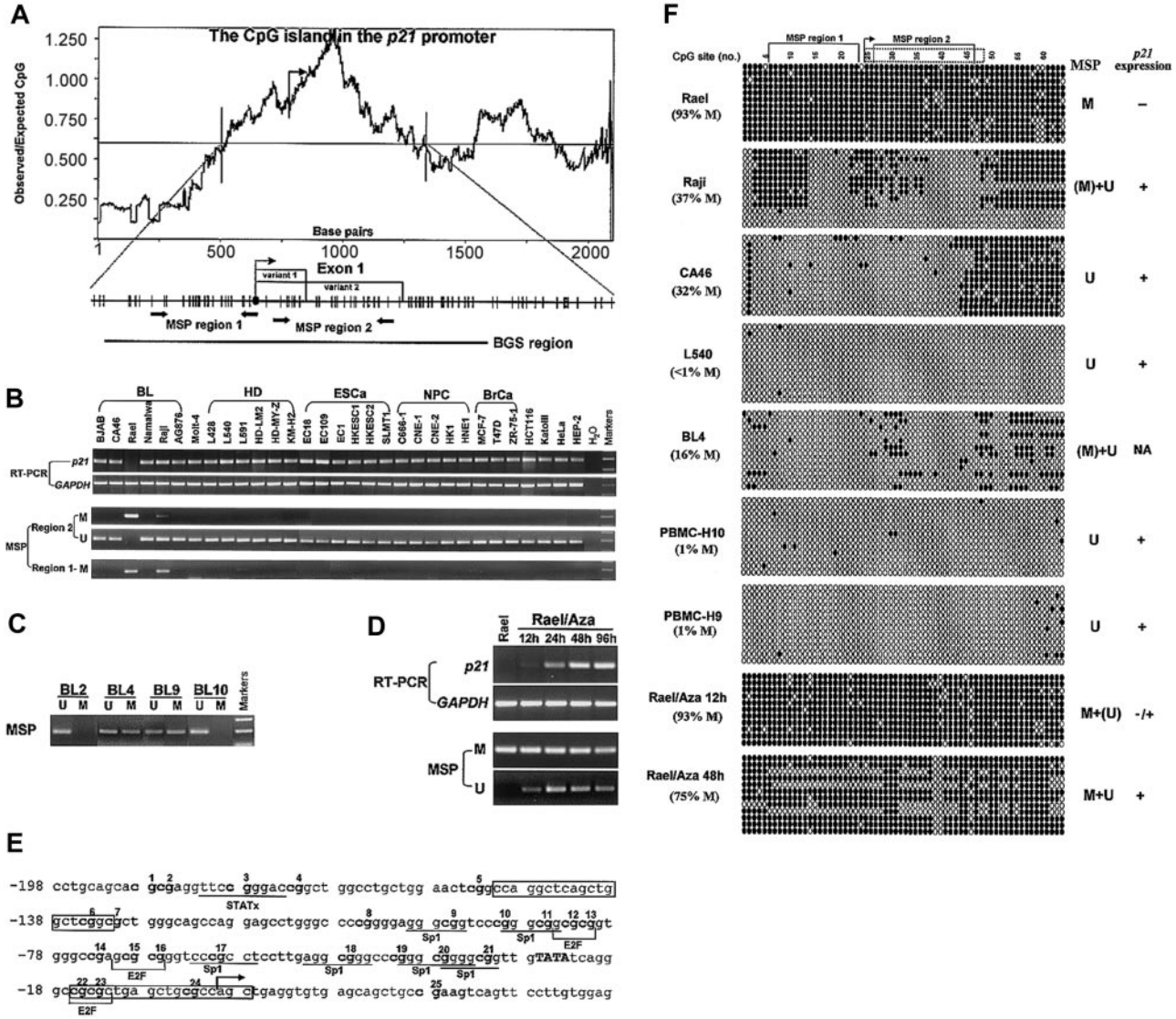

Figure 1. Expression and methylation status of *p21* in various cell lines and primary tumors. (A) The CpG island in the *p21* promoter includes the core promoter, exon 1 (with 2 splicing variants) and part of intron 1. A DNA region with an observed/expected CpG ratio of more than 0.6 and a GC content of more than 50% is considered a CpG island.[10] The transcription start site is indicated by bent arrows (based on NCBI database). The 2 discrete MSP regions and one BGS region analyzed in the p21 CGI are indicated. Region 1 corresponds to the area studied by Roman-Gomez et al[2] and Shen et al,[3] while region 2 has also been studied by Shen et al. MSP primers used are as follows, for region 1 (methylated), p21m1: 5′-TTAGGTTTAGTTGGTTCGGC, p21m2: 5′-ACTAACGCAACTCAACGCG; for region 2 (methylated), p21bm1: 5′-GTGAACGTAGTATATATTCGC, p21bm2: 5′-ATAAAACCGAAACTAAACGCG; and for region 2 (unmethylated), p21bu1: 5′-TTGTGAATGTAGTATATATTTGT, p21bu2: 5′-TTATAAAACCAAAACTAAACACA. Primers for BGS are as follows: p21BGS1, 5′-AGGGAAGTGTTTTTTTGTAGT and p21BGS2, 5′-TAACCAAAAATTCCTATACTTA. MSP primers have been tested for not amplifying any unbisulfited DNA. MSP and BGS were performed as previously described.[10] (B) Representative semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and MSP results of *p21* in cell lines. *GAPDH* was used as a control for RT-PCR.[10] M indicates methylated; U, unmethylated; EsCa, esophageal carcinoma; NPC, nasopharyngeal carcinoma; BrCa, breast carcinoma. (C) Representative MSP results in several primary BLs. (D) Demethylation and activation of *p21* in Rael after treatment with 5 μM 5-aza-2′-deoxycytidine (Aza). Hours of treatment are indicated by 12 h, 24 h, 48 h, and 96 h. (E) Partial sequence of the *p21* promoter CGI. CpG sites are bolded. The transcription start site is marked as a bent arrow. The TATA box is capitalized. Six Sp1 binding sites and some other regulatory elements (E2F, STAT) within this sequence are underlined. MSP primers for region 1 are framed. (F) High-resolution methylation analysis of the *p21* promoter by BGS, which reveals the methylation status of every CpG site in the studied region. A 576-bp region spanning the *p21* promoter with 64 CpG sites was analyzed, with the 2 MSP regions labeled. The *p21* exon 1 is labeled by a dot-framed box. Each CpG site is shown at the top row as a number. BGS results of 4 lymphoma cell lines (Rael, Raji, CA46, and L540), 2 normal PBMCs, and 1 primary BL are shown. Each row in the grid, next to the sample name, represents an individual allele of the *p21* promoter analyzed by BGS in that sample.[10] Filled circles are methylated CpG sites and open circles are unmethylated CpG sites. % M is the percent of methylated CpG site of all CpG sites analyzed. M indicates methylated; U, unmethylated; (M), weakly methylated; (U), weakly unmethylated; NA, not available.

Table 1. Summary of *p21* promoter methylation in various cell lines and primary tumors

	Methylation (%)	Method used	Reference
Present study		MSP + BGS	NA
Tumor cell lines			
Burkitt lymphoma	2/7 (29)		
Hodgkin disease	0/6		
Leukemia	0/4		
Nasopharyngeal Ca	0/10		
Esophageal Ca	0/16		
Breast Ca	0/3		
Laryngeal Ca	0/1		
Lung Ca	0/1		
Colorectal Ca	0/5		
Gastric Ca	0/1		
Hepatocellular Ca	0/2		
Cervical Ca	0/2		
Primary tumors (geographic origin)			
Burkitt lymphoma (Africa)	2/8 (25)		
Diffuse large B-cell lymphoma (United Kingdom)	0/13		
Follicular lymphoma (United Kingdom)	0/6		
Mantle cell lymphoma (United Kingdom)	0/4		
Posttransplant lymphoma (United States)	0/13		
Anaplastic large cell lymphoma (United Kingdom)	0/4		
Nasal NK/T cell lymphoma (Hong Kong)	1/18 (6)		
Hodgkin disease (United States, United Kingdom)	0/31		
Other types of lymphoma (United Kingdom)	0/3		
Nasopharyngeal Ca (Taiwan, Singapore)	0/25		
Esophageal Ca (Hong Kong)	0/35		
Breast Ca (Singapore)	0/21		
Gastric Ca (United Kingdom)	0/5		
Lung Ca (Singapore)	0/1		
Normal PBMC (Singapore)	0/12		
Normal tissues (lymph node, breast, placenta, pancreas)	0/10		
Literature review			
Cell lines and primary tumors			
Hematopoietic tumor cell lines	0/14	COBRA	4
Central nervous system lymphoma	0/18	MSP	5
Acute lymphocytic leukemia	51/124 (41)	RE/PCR	2
Acute lymphocytic leukemia	0/31	COBRA + MSP	3
Gastric lymphoma	4/13 (31)	MSP	9
Instestinal lymphoma	0/14	MSP	9
Lung Ca cell lines	2/16 (13)	COBRA	6
Colorectal Ca cell lines	0/10	COBRA	4
Gastric Ca cell lines	0/15	COBRA	4,8
Hepatocellular Ca cell lines	0/5	COBRA	4
Pancreatic Ca cell lines	0/2	COBRA	4
Prostate Ca	2/16 (13)	MSP	7
Rhabdomyosarcoma cell lines and tumors	15/31 (48)	RE/Southern blot	1

NA indicates not applicable; Ca, carcinoma; COBRA, combined bisulfite restriction analysis; and RE, restriction enzyme digestion.

(Figure 1C). We also examined *p21* methylation in more detail by bisulfite genomic sequencing (BGS; Figure 1E-F).[10] Consistent with our MSP analysis, the results showed that, in Rael, 93% CpG sites were methylated, whereas only 37% CpG sites (most of them outside the core promoter) were methylated in Raji. Only a few scattered CpG sites were methylated in other samples. The abundant expression of *p21* in CA46 suggested that the patchy methylation of *p21* in intron 1, but not the core promoter and exon 1, does not affect its expression.

We also treated Rael with 5-aza-2′-deoxycytidine. *p21* expression was restored after 24 hours of treatment, and more profoundly at 48 hours and 96 hours. Concomitantly, unmethylated *p21* alleles were detected after the treatment (Figure 1D). Therefore, this promoter could be demethylated and activated by 5-aza-2′-deoxycytidine alone, indicating that methylation directly mediates its suppression.

Furthermore, we have reviewed all literature about *p21* methylation (Table 1). *p21* methylation is rare in tumors in general but does occur in certain tumors. The reports using restriction enzyme digestion–based assays, which only detect the methylation of very few CpG sites at specific restriction sites, tend to detect relatively high frequencies of *p21* methylation. The CpG within a signal transducers and activators of transcription (STAT) site at −692 in the distal promoter was reported frequently methylated in Rhabdomyosarcoma and normal tissues[1]; however, we did not detect any methylation in any normal tissue or PBMCs. Studies using bisulfite-modification–based methods (MSP and combined bisulfite restriction analysis [COBRA]) tend to detect little if any methylation. The different results of the 2 recent reports may also be due to their different techniques, or a geographic/ethnic variation as suggested by the authors.[2,3] With this precaution, we

have used both MSP and BGS to verify our results, and recruited samples from all over the world.

In summary, we found that *p21*, unlike *p16* and *p15*,[5] is rarely inactivated by methylation in lymphomas and carcinomas. However, our study still does not rule out the possibility of epigenetic repression of this gene, to some extent, through chromatin/histone structure changes, since histone deacetylase inhibitors trichostalin A (TSA), phenylbutyrate, and subercylanilide hydroxamic acid (SAHA) can also activate *p21* expression.[11] 5-aza-2′-deoxycytidine can also activate *p21* expression through methylation-independent mechanisms.

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To the editor:

Enough already of the word "robust"!

Lately, I have noticed that the word "robust" has become one of the overused terms in biomedical science, and we hematologists are among the worst offenders. This abuse of "robust" is a relatively recent phenomenon; the number of biomedical articles published annually with the word "robust" in title or abstract has increased 40-fold since 1982 (Figure 1A). The occurrence of "robust" in *Blood* articles has gone from nil in the early 1990s to more than 100 references in 2003 (Figure 1B). While other terms have also experienced dramatic growth (eg, *p53*), such usage changes have occurred because of scientific discovery, not linguistic conformity and imitation.

Dictionary definitions of "robust" (derived from a Latin word for "oak") include hale and hearty synonyms like "vigorous" and "firm." Describing an object or idea as "robust" implies that it has the power to withstand physical or intellectual challenge. But in modern medical parlance, this once useful code word is becoming meaningless jargon. "Robust" used to designate a treatment strategy or laboratory technique that actually worked most of the time, in contrast to those that failed frequently and for no apparent reason. When a PhD described an assay as "robust," the audience could safely assume that the technique was so straightforward,

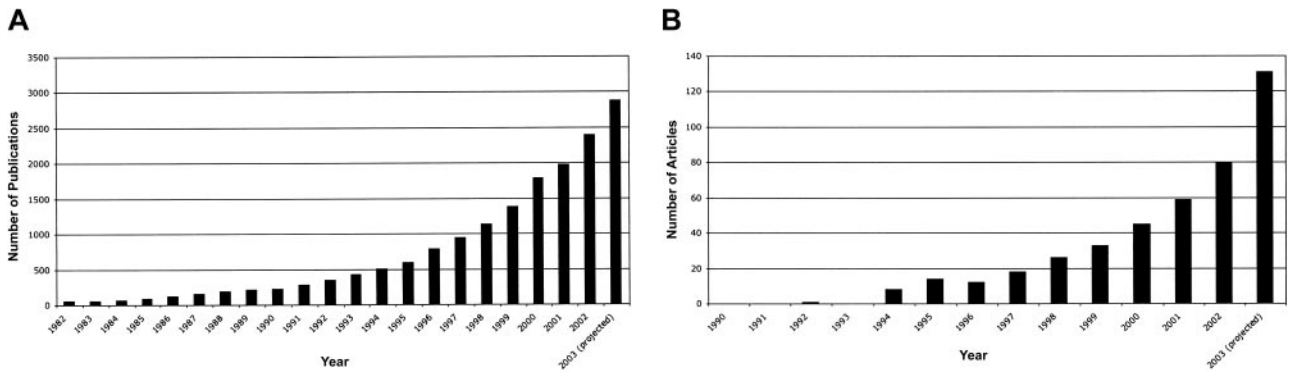

Figure 1. Growth of usage of the word "robust" in recent publications. (A) The number of biomedical publications each year containing the word "robust" in the title or abstract, indexed by the National Library of Medicine and accessed via PubMed at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed on October 20, 2003. Publications that included the word "robust" only in the author field (eg, publications by Drs Robusto, Robustellini, Robustelli, and Robustova) were excluded. Although PubMed searches also retrieve a small subset of paleontology and anthropology articles, reading the recent abstracts verified that the growth of articles containing the word "robust" has not come because research concerning *Australopithecus robustus* and related East African hominids is proceeding at an astonishing pace. The total annual number of PubMed-indexed publications increased less than 2-fold from 1982 to 2002. Figures for 2003 are projected based on occurrences of robust through October 1. (B) The number of articles published in *Blood* each year that have included the word "robust" in the abstract, title, or text of the article. The full text of all *Blood* articles published since January 1990 was searched at http://www.bloodjournal.org/search.dtl on October 20, 2003.