To the editor:

## Sorting zebrafish thrombocyte lineage cells with a Cd41 monoclonal antibody enriches hematopoietic stem cell activity

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An understanding of hematopoietic stem cell (HSC) biology is therapeutically important because of the many human diseases that result from errors in HSC regulation or that can be treated with HSC transplantation. The zebrafish is a powerful model for studying HSC biology in vivo[1-3]; however, monoclonal antibodies are challenging to generate for use with zebrafish hematopoietic cells.[4] Here, we report the development and characterization of a monoclonal antibody to zebrafish Cd41 and show that it can be used to negatively enrich for HSCs.

Zebrafish Cd41, also known as GpIIb and Itga2b, is a cell-surface protein expressed at high levels on thrombocytes and at lower levels on HSCs.[5-7] In humans and other species, CD41 forms cell-surface heterodimers with CD61, also known as GPIIIa or ITGB3. The CD41/CD61 (GPIIb/IIIa) heterodimer plays a critical role in platelet activation, and deficiencies in its function cause the human bleeding disorder Glanzmann thrombasthenia.[8,9] Previous work has shown that *Tg(cd41:EGFP)* zebrafish can be used to positively enrich for HSCs.[6,10] In adult zebrafish, *cd41:EGFP*$^{Low}$ cells in kidney marrow have HSC activity, whereas *cd41:EGFP*$^{High}$ cells in peripheral blood are thrombocytes.[6] These data suggested that an antibody to Cd41 could be useful for studying the zebrafish thrombocyte lineage and for isolating zebrafish HSCs.

To generate a monoclonal antibody to zebrafish Cd41, in 293T cells we expressed a secreted form of zebrafish Cd41/mouse CD61 in which the transmembrane domains of each protein were replaced by an acid peptide and base peptide, respectively, to promote leucine zipper-mediated heterodimerization and secretion. The heterodimeric protein was then used as an immunogen in the production of mouse hybridomas (see supplemental Figure 1A, available on the *Blood* Web site). Hybridomas were screened using a cell line expressing native zebrafish Cd41 and zebrafish Cd61b. The Cd61b was FLAG-tagged to allow confirmation of heterodimer expression on the cell surface without altering the Cd41 epitope. This yielded a reactive hybridoma that was selected for further subcloning and characterization (supplemental Figure 1B).

We initially tested the Cd41 antibody by flow cytometry using peripheral blood from adult *Tg(cd41:EGFP)* zebrafish. This revealed that the Cd41 antibody recognizes *cd41:EGFP*-positive cells with greater than 99% sensitivity and specificity (Figure 1A). Based on light scatter characteristics, these cells are predominantly located in the expected region,[5] which has traditionally been termed the "lymphoid" gate (Figure 1B).[11] At least 2 discrete populations of double-positive cells are discernable; the most abundant of these is relatively restricted to the lymphoid gate. Cytopreparations of cells sorted by flow cytometry demonstrated that Cd41 antibody-positive cells are predominantly thrombocytes, as anticipated (Figure 1C). Furthermore, RNA sequencing confirmed that the most highly expressed genes in antibody-positive cells/zebrafish thrombocytes are well known to be expressed by human and mouse platelets and that there is significant evolutionary conservation among the 3 species (supplemental Tables 1 and 2; supplemental Figure 2). As an alternative method to flow cytometry, the characteristics of the Cd41 antibody were assessed by immunofluorescence (supplemental Figure 3); congruent with the flow cytometry results, the Cd41 antibody stained cells in immunofluorescence experiments with greater than 95% sensitivity and specificity.

To confirm that the Cd41 antibody recognizes zebrafish Cd41, we tested the antibody against a *cd41* mutant zebrafish (*itga2b*$^{sa10134}$) that is expected to lack expression of Cd41.[12] Homozygous mutant zebrafish are observed at much less than the expected frequency in heterozygous crosses but have circulating thrombocytes. Analysis of peripheral blood from *cd41*$^{-/-}$ zebrafish by flow cytometry revealed a loss of Cd41 antibody staining (Figure 1D). The proportion of peripheral blood cells stained was normal in heterozygous mutant zebrafish (Figure 1D), but the median fluorescent intensity of heterozygous cells was two-thirds that of the wild-type cells (Figure 1E). This is consistent with decreased cell-surface expression of Cd41 in heterozygous cells. Because loss of Cd41 at the cell surface might compromise Cd61b cell-surface expression, we confirmed that the Cd41 antibody binds to Cd41 and not Cd61b in the Cd41/Cd61b heterodimer using adult *Tg(cd61b:EGFP)* zebrafish (Figure 1F).

Having characterized the Cd41 antibody for use with adult zebrafish peripheral blood, we turned our attention to adult zebrafish kidney marrow. In kidney marrow, the Cd41 antibody identifies a subset of *cd41:EGFP*-positive cells, specifically the population with higher *cd41:EGFP* expression (Figure 2A). Multiple possibilities for this differential binding exist, including transgene activation in the absence of transcription at the endogenous locus and production of discrete epitopes in the 2 cell groups. Because some Cd41 antibody-positive cells isolated from kidney marrow in the absence of a significant forward scatter size limit appeared to be immature when examined using cytopreparations, and because a minority are located within the

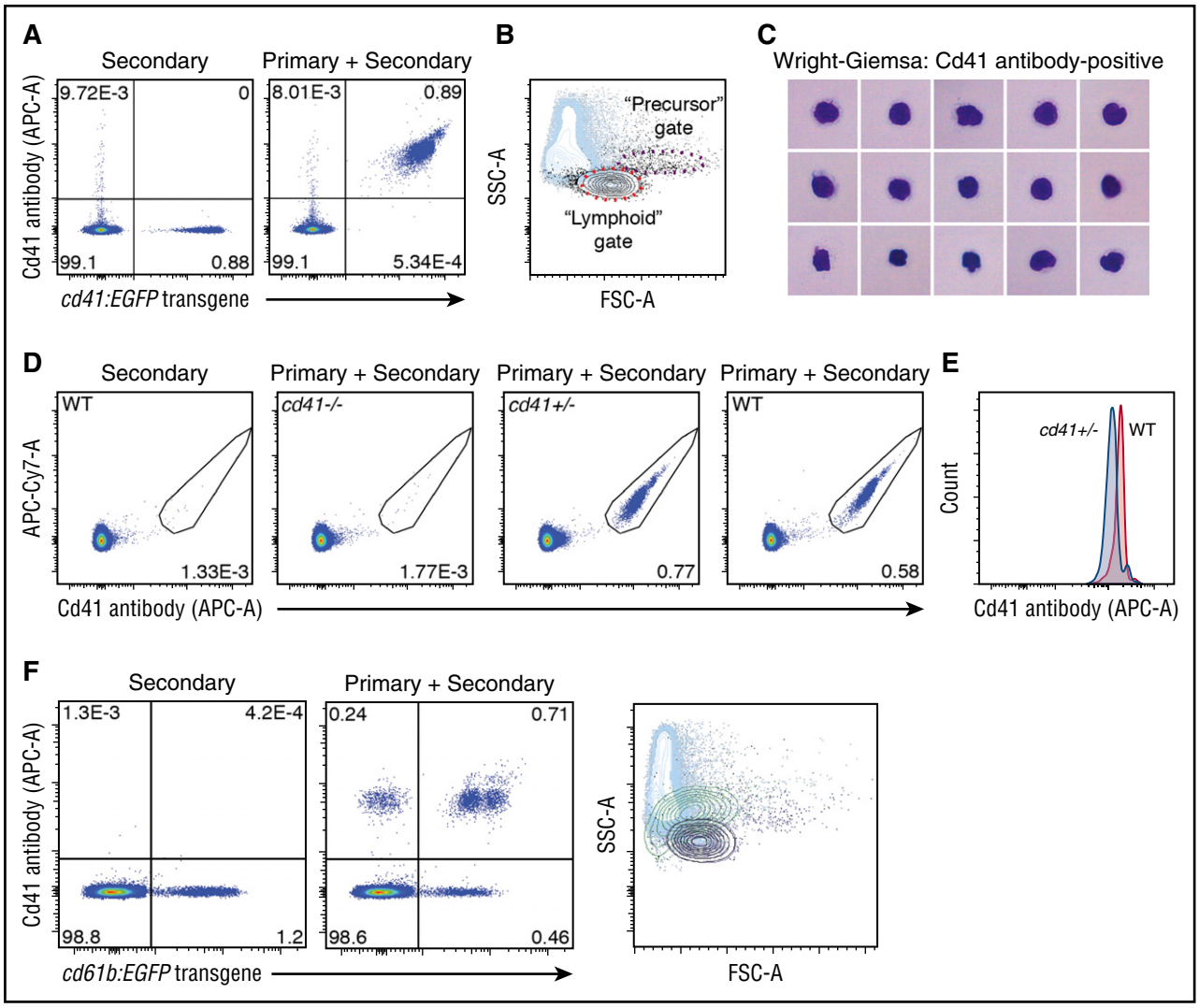
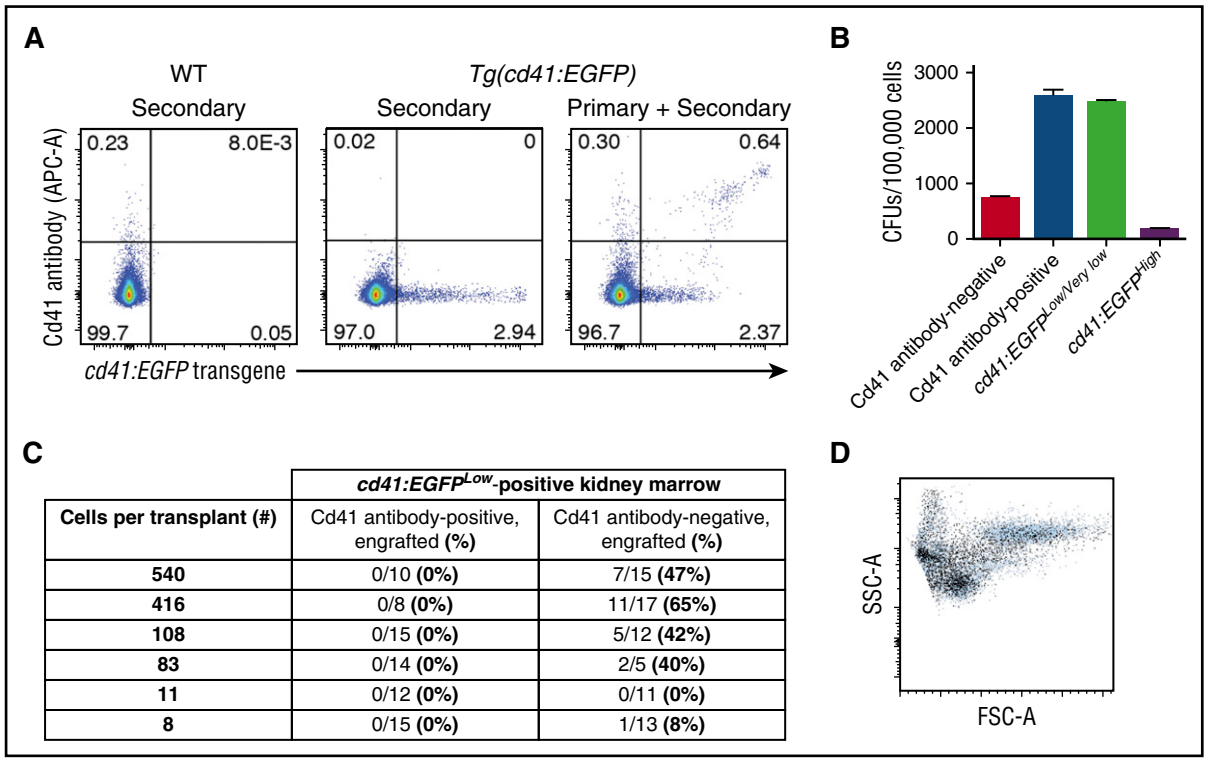



**Figure 1. The Cd41 antibody binds *cd41:EGFP*-positive cells in zebrafish peripheral blood with >99% sensitivity and specificity and is specific for Cd41.** (A) The Cd41 antibody recognizes *cd41:EGFP*-positive cells in zebrafish peripheral blood with >99% sensitivity and specificity by flow cytometry. (B) Light scatter characteristics demonstrate that double-positive cells (black) are mostly located within the "lymphoid" gate (red dashed line) when overlaid on live cells (blue). Contour levels are set at 10% and outliers are shown. The "precursor" gate is also shown for reference (purple dashed line). (C) Wright-Giemsa stain of cytopreparation showing that Cd41 antibody-positive cells are generally thrombocytes based on morphology. (D) Flow cytometry of zebrafish peripheral blood reveals a loss of Cd41 antibody staining in *cd41* mutant zebrafish. There is no reduction in the percentage of antibody-stained Cd41-positive cells in heterozygous zebrafish. (E) The median fluorescence intensity of cells from heterozygous zebrafish is about two-thirds that of wild-type (WT) zebrafish. (F) In spleen cells from adult zebrafish, *cd61b:EGFP* is expressed by a subset of Cd41 antibody-positive cells, but populations of both *cd61b:EGFP* single-positive and Cd41 antibody single-positive cells also exist. By light scatter characteristics, the *cd61b:EGFP* single-positive cells (green) map to a slightly different location than the overlapping double-positive cells (black) and Cd41 antibody single-positive cells (purple). Live cells are shown in the background (blue). Contour levels are set at 10% and outliers are shown. APC-A, allophycocyanin-area; FSC-A, forward scatter-area; SSC-A, side scatter-area.

so-called "precursor" gate, we performed colony-forming assays to determine if some of these cells were progenitor cells that had not yet terminally differentiated. This revealed that Cd41 antibody-positive cells exhibit colony-forming activity comparable to what is obtained with *cd41:EGFP*$^{Low/Very\ Low}$-positive cells (Figure 2B). In this case, the lower level of colony-forming activity in the Cd41 antibody-negative cell population reflects the gating strategy, which includes all Cd41 antibody-negative cells: although HSCs and progenitors such as common myeloid progenitors, common lymphoid progenitors, and granulocyte-monocyte progenitors would be included, abundant terminally differentiated cells such as mature myeloid cells, lymphoid cells, and red blood cells are also included. As expected, *cd41:EGFP*$^{High}$ cells do not form significant numbers of colonies because they are largely mature thrombocytes.[5,6]

Because the *cd41:EGFP*$^{High}$ population is enriched for thrombocytes and the *cd41:EGFP*$^{Low}$ population is enriched for HSCs,[6] we sought to determine whether the Cd41 antibody permits enrichment of adult zebrafish HSCs by negative selection. We performed limiting dilution transplantation experiments using kidney marrow from *Tg(cd41:EGFP;ubi:mCherry)* zebrafish that was sorted into a *cd41:EGFP*$^{Low}$, Cd41 antibody-positive fraction and a *cd41:EGFP*$^{Low}$, Cd41 antibody-negative fraction. The sorting strategy included a restriction on forward scatter and used splenocytes to help more rigorously define the *cd41:EGFP*$^{High}$ population (supplemental Figure 4A). Morphologically, the Cd41 antibody-positive fraction contained thrombocytes (supplemental Figure 4B) and the Cd41 antibody-negative fraction contained cells that were mostly immature with high nuclear to cytoplasmic ratios (supplemental




| Cells per transplant (#) | *cd41:EGFP*$^{Low}$-positive kidney marrow | |
|---|---|---|
| | Cd41 antibody-positive, engrafted **(%)** | Cd41 antibody-negative, engrafted **(%)** |
| **540** | 0/10 **(0%)** | 7/15 **(47%)** |
| **416** | 0/8 **(0%)** | 11/17 **(65%)** |
| **108** | 0/15 **(0%)** | 5/12 **(42%)** |
| **83** | 0/14 **(0%)** | 2/5 **(40%)** |
| **11** | 0/12 **(0%)** | 0/11 **(0%)** |
| **8** | 0/15 **(0%)** | 1/13 **(8%)** |

**Figure 2. The Cd41 antibody binds thrombocytes and thrombocyte precursors in zebrafish kidney marrow and permits HSC enrichment.** (A) The Cd41 antibody binds *cd41:EGFP*$^{High}$-positive cells (including thrombocytes) preferentially in kidney marrow. (B) Colony-forming assays suggest that Cd41 antibody-positive cells in kidney marrow include thrombocyte precursors. (C) Transplantation of sorted *cd41:EGFP*$^{Low}$ kidney marrow cells reveals that the Cd41 antibody-positive cell fraction does not engraft, but that the Cd41 antibody-negative cell fraction does. (D) Representative flow cytometry plot from the kidney marrow of a transplant recipient at 3 months posttransplant. Multilineage engraftment is apparent based on the location of *ubi:mCherry*-positive cells (black) relative to live cells (blue) by light scatter characteristics.

Figure 4C). These fractions were transplanted into irradiated recipients and engraftment was assessed at 3 months posttransplant. Engraftment was never obtained from *cd41:EGFP*$^{Low}$, Cd41 antibody-positive fractions; by contrast, engraftment was frequently obtained from *cd41:EGFP*$^{Low}$, Cd41 antibody-negative fractions (Figure 2C). Engraftment was multilineage based on expression of the *ubi:mCherry* transgene in erythroid, myeloid, precursor, and lymphoid gates by flow cytometry (Figure 2D).

The Cd41 antibody characterized in this study is the first monoclonal antibody to permit HSC enrichment by flow cytometry in zebrafish. Limiting dilution software suggests that the frequency of HSCs in our *cd41:EGFP*$^{Low}$, Cd41 antibody-negative fraction is 1/456 (302-688) but this is likely an underestimate resulting at least in part from transplantation being performed without matching major histocompatibility loci between donors and recipients. Our results support the broad use of the Cd41 antibody with other transgenic zebrafish lines in the quest to purify zebrafish HSCs. Indeed, the Cd41 antibody represents a first step toward developing a set of zebrafish "lineage-negative" markers that permit the enrichment of HSCs, as is already done in mice and humans.

*J.M.G. and A.D.L. contributed equally to this study.

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**Contribution:** J.M.G. performed fluorescence-activated cell sorting (FACS) experiments, transplantation, cytopreparations, immunofluorescence, and RNA sequencing; generated the first draft of the manuscript; and incorporated feedback from the other authors into subsequent drafts of the manuscript. A.D.L. was responsible for generating the Cd41 antibody and performed FACS experiments. M.S. performed the bioinformatics analyses. M.C.B. performed immunofluorescence experiments and helped with transplantation experiments. M.B.A. helped with transplantation experiments. E.M.D. helped with the generation of the Cd41 antibody. B.B. helped with the generation of the Cd41 antibody. R.I.H. provided technical advice. D.L.S. performed the colony-forming assay. C.L. provided technical advice and reagents for expressing secreted heterodimers of Cd41 and CD61. T.A.S. provided technical advice and reagents for expressing secreted heterodimers of Cd41 and CD61. L.I.Z. guided the research and edited the manuscript.

**Conflict-of-interest disclosure:** L.I.Z. is a founder and/or on the board of directors and has equity in Fate Therapeutics, Marauder Therapeutics, and Scholar Rock. The remaining authors declare no competing financial conflicts.

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# To the editor:

## Mutational landscape and response are conserved in peripheral blood of AML and MDS patients during decitabine therapy

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Quantitative response evaluation in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDSs) relies on the morphologic quantification of bone marrow (BM) blasts. This process is subject to the operator-dependent quality of BM collection and the interobserver variability among pathologists.[1-9] Determining responses can be further complicated by hemodiluted sampling, declined BM biopsies, dry taps, and confounding drug toxicities that prevent count recovery or give the impression of persistent dysplasia.

We previously observed that the clonal architecture of diagnostic BM samples in AML patients with leukocytosis is recapitulated in their simultaneously obtained peripheral blood (PB).[10] Other groups have observed concordant PB *NPM1* mutation clearance and PB copy number abnormalities[11-14] and have occasionally used PB for mutation discovery.[15-17] Therefore, we sought to compare the mutation burden in paired serial PB and BM samples in patients with AML or MDS to determine whether sequencing of PB samples is a viable approach for determining clonal architecture and whether it might provide an adjunct, and less invasive, measure of response to therapy.

We quantified mutation burden in PB vs BM samples in a subset of patients treated at Washington University with 10-day courses of decitabine (NCT01687400).[18] Twenty-seven patients were selected: 22 with AML and 5 with MDS. Cases were selected based on the presence of at least 2 somatic mutations in the BM sample from each patient using a panel of 264 recurrently mutated AML genes (see supplemental Table 1, available on the *Blood* Web site) and adequate DNA from matched PB samples at multiple times. PB DNA was analyzed using this recurrently mutated AML gene panel (supplemental Methods; Welch et al[18] and Cancer Genome Atlas Research Network[19]). In total, 138 somatic mutations were detected (median of 4 mutations per patient) across 93 time points (median of 3 time points per patient), providing a total of 446 pairwise comparisons of mutation detection in the blood vs marrow. The median white blood cell count across all time points was 1500/μL (range, 100-75 000/μL). The median age of the patients was 73 years (range, 47-88). Clinical responses included 5 complete remissions, 9 complete remissions with incomplete count recovery, 2 marrow complete remissions, 3 partial remissions, 6 stable disease, and 2 progressive disease. The median read depth in PB samples was ×193; in BM samples, the median read depth was ×295. All patients consented to genome sequencing analysis and were treated in accordance with the Declaration of Helsinki.

Mutation patterns observed in the PB strongly paralleled the BM results, including subclonal architecture (eg, 1012 and 1018), copy number variation (eg, 1038 and 1019), dynamic responses during decitabine therapy (eg, 1009 and 1048), early expansion of relapse subclones (eg, 1009 and 1021), and clonal hematopoiesis during remission unrelated to the malignant clone[20] (eg, 1014) (Figure 1; supplemental Figures 1-4).

The sensitivity and specificity of PB sequencing to detect BM mutations from the same time point were calculated across the entire dataset. For BM mutations with variant allele frequencies (VAFs) >5% and read counts >100, PB sensitivity was 88% and specificity was 84%. A receiver operator curve was generated using a range of VAF and read-count thresholds (supplemental Figure 5A-B); the area under the curve increased only modestly with higher read-count thresholds (0.934 vs 0.943 for read-count cutoffs of 50 vs 200; VAFs >5%).

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