HES6 knockdown in human hematopoietic precursor cells reduces their *in vivo* engraftment potential and their capacity to differentiate into erythroid cells, B cells, T cells and plasmacytoid dendritic cells

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Abstract

Hematopoiesis is driven by molecular mechanisms that induce differentiation and proliferation of hematopoietic stem cells and their progeny. This involves the activity of various transcription factors, such as members of the Hairy/Enhancer of Split (HES) family, and important roles for both HES1 and HES4 have been shown in normal and malignant hematopoiesis. Here, we investigated the role of HES6 in human hematopoiesis using *in vitro* and *in vivo* models. Using bulk and single-cell RNA-sequencing data, we show that *HES6* is expressed during erythroid/megakaryocyte and plasmacytoid dendritic cell development, as well as in multipotent precursors and at specific stages of T- and B-cell development following pre-B-cell receptor and pre-T-cell receptor signaling, respectively. Consistently, knockdown of *HES6* in cord blood-derived hematopoietic precursors in well-defined *in vitro* differentiation assays resulted in reduced differentiation of human hematopoietic precursors towards megakaryocytes, erythrocytes, plasmacytoid dendritic cells, B cells and T cells. In addition, *HES6* knockdown hematopoietic stem and progenitor cells displayed reduced colony-forming unit capacity *in vitro* and impaired potential to reconstitute hematopoiesis *in vivo* in a competitive transplantation assay. We demonstrate that loss of *HES6* expression has an impact on cell cycle progression during erythroid differentiation and provide evidence for potential downstream target genes that affect these perturbations. Thus, our study provides new insights into the role of HES6 in human hematopoiesis.

Introduction

The vast majority of hematopoietic cells in the human body arise from multipotent hematopoietic stem and progenitor cells (HSPC) that reside in the bone marrow. The differentiation of these HSPC into mature blood cells is the result of successful integration of external stimuli and cell-intrinsic mechanisms that lead to chromatin remodeling and changes in gene expression, thereby activating and repressing specific molecular pathways.[1,2] Research on normal hematopoiesis is important to increase knowledge on stem cell function and differentiation. This will facilitate blood cell regeneration and serve as a reference to uncover mechanisms that induce hematologic malignancies. While animal models are instrumental to advance this field, human studies provide a more direct translational perspective and the opportunity to reveal human-specific mechanisms. The Notch signaling pathway is an important regulator of normal hematopoiesis and often involved in leukemia.[3-6] Notch activation induces expression of downstream target genes, including members of the Hairy/Enhancer of Split (HES) family of transcription factors that contains seven members in humans (*HES1-7*). While important roles for HES1, HES4 and HES5 have been described in hematopoiesis,[7-9] the potential involvement of HES6 in hematopoietic stem cell differentiation has remained unclear. During embryonic development, HES6 is implicated in mesoderm specification and neurogenesis in which it can inhibit the

transcriptional repression activity of HES1.[10-12] *HES6* also acts as an oncogene in different types of cancer including colorectal, prostate and gastric cancer, as well as melanoma and glioma.[13-18] In these malignancies, it mainly drives metastatic disease and proliferation.[13,15,17] In a hematologic context, *HES6* appears overexpressed in blastic plasmacytoid dendritic cell neoplasm.[19,20] And while HES6 had been proposed to mediate erythropoiesis,[21,22] only recently it was shown to be a cofactor for GATA1 during erythroid development.[23] In this study, we investigated the functional role of HES6 in human hematopoiesis more broadly using both *in vitro* and *in vivo* models and found that, in addition to a role in erythropoiesis, *HES6* is also important to support the *in vivo* reconstitution potential of human HSPC, and the capacity of HSPC to differentiate into erythrocytes, B cells, T cells and plasmacytoid dendritic cells (pDC).

Methods

Isolation of CD34$^+$ hematopoietic precursor cells and transduction

Human cord blood was obtained from the Hematopoietic Cell Biobank UZ Ghent with informed consent and according to the Medical Ethical Committee of Ghent University Hospital (EC protocol: EC-Bio/1-2018/sds, EC/2021-010/sds, EC/2021-020/sds, EC/2019/0826). CD34$^+$ HSPC were processed as described elsewhere[9] and cultured (density of 5x10^5 cells/mL) for 2 days in medium that depended on the further experimental set-up (*Online Supplementary Table S1*). Subsequently, cells were transduced for 2 days with lentivirus using Retronectin (TAKARA; #T100B).

Other methods are available in the *Online Supplementary Material and Methods*.

Results

HES6 is required during early T-cell development

Notch signaling initiates T-cell development and induces expression of downstream target genes, including members of the HES family. Bulk RNA-sequencing data[24] confirmed that the Notch targets *HES1* and *HES4* are expressed during early T-cell development until the β-selection checkpoint. In contrast, *HES6* expression was only detected after β-selection (*Online Supplementary Figure S1A*). Consistently, *HES6* did not show Notch-dependent transcriptional activation in hematopoietic progenitors, in contrast to *DTX1* and *HES1* (*Online Supplementary Figure S1B, C*).[25,26] We further explored *HES1* and *HES6* expression in a published single-cell RNA-sequencing dataset[27] (*Online Supplementary Figure S1D*) and confirmed *HES1* expression in the immature stages of T-cell development (*Online Supplementary Figure S1E*). Similar to the results of bulk RNA sequencing, *HES6* expression peaked in the double-positive stages after β-selection, but we also observed expression in immature, proliferating CD4$^-$CD8$^-$ double-negative thymocytes (Figure 1A). Thus, single-cell RNA sequencing reveals that *HES6* is mainly expressed in proliferating thymocytes (*Online Supplementary Figure S1F, G*).

To study the function of HES6 in human T-cell development, we used lentiviral-based short hairpin (sh)RNA-mediated knockdown and cultured transduced HSPC in an *in vitro* OP9-DLL4 T-cell differentiation assay[28] (*Online Supplementary Figure S2A*). All three shRNA constructs significantly decreased *HES6* expression compared to that in control shRNA-transduced HSPC, with the knockdown ranging from 55% to 75% (Figure 1B). While the co-culture started with a sorting purity of >95% transduced cells (BFP$^+$), the frequency of BFP$^+$ cells at day 21 was significantly lower in *HES6* knockdown cultures compared to the control (*Online Supplementary Figure S2B, C*). *HES6* knockdown consistently reduced the absolute number of BFP$^+$ cells compared to that in control transduced cultures (*Online Supplementary Figure S2D*). After 14 days, the frequencies of HSPC (CD34$^+$CD7$^-$) and T-cell precursors (CD34$^+$CD7$^+$) were increased following *HES6* knockdown but there was an inconsistent impact on the cell numbers of those populations (Figure 1C, *Online Supplementary Figure S2E, F*). At this time point, the frequencies and total cell numbers of more differentiated T-cell precursor cells (CD34$^-$CD7$^+$) were significantly reduced (Figure 1C, *Online Supplementary Figure S2E, F*). After 21 days of co-culture, *HES6* knockdown decreased differentiation from CD7$^+$CD5$^-$ cells to T-cell specified precursor cells (CD7$^+$CD5$^+$), resulting in reduced total numbers of T-cell specified precursor cells (Figure 1D, *Online Supplementary Figure S2G, H*). Furthermore, we observed reduced frequencies and absolute cell numbers for CD4$^+$CD8b$^-$ as well as CD4$^+$CD8b$^+$ cells after *HES6* knockdown compared to control (Figure 1E, *Online Supplementary Figure S2I, J*). In conclusion, *HES6* knockdown inhibits the development of T-cell specified precursor cells as well as double-positive cells, in agreement with its expression in proliferating double-negative cells and β-selected developing thymocytes.

Stage-specific role for HES6 during human B-cell development

To further explore the role of HES6 in hematopoiesis, we analyzed published single-cell RNA-sequencing data of human bone marrow[29] and observed *HES6* expression in natural killer (NK) cells, pDC, immature B cells and, in particular, erythroid cells (Figure 2A). Using a bone marrow dataset,[30] with distinct stages of human B-cell development annotated (Figure 2B), we discovered *HES6* expression in proliferating cells (*Online Supplementary Figure S3A*) and particularly in large, cycling pre-B-cells as confirmed by expression of the proliferation marker *MKI67* (Figure 2C). To study HES6 in B-cell development, we cultured shRNA-transduced HSPC in an *in vitro* B-cell differentiation

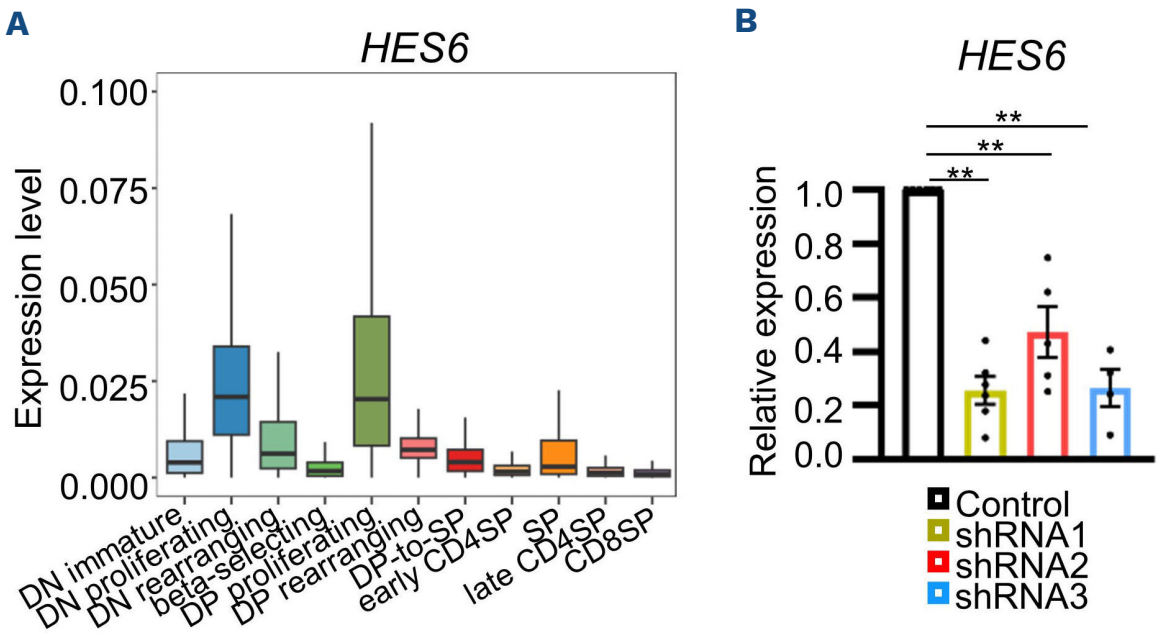

Figure 1. *HES6* is important for early *in vitro* T-cell development. (A) Boxplots derived from single-cell RNA-sequencing data showing pseudo-bulk log-normalized imputed mRNA expression levels of *HES6* across different stages of human postnatal T-cell development.[27] Boxplots show the median and first and third quartiles. (B) Quantitative real-time polymerase chain reaction analysis of *HES6* in cord blood CD34⁺Lin⁻BFP⁺ cells transduced with a control short hairpin (sh)RNA or three different *HES6* shRNA, normalized to housekeeping genes (*ACTB*, *B2M*, *SDHA*) and relative to the expression in the control shRNA condition (shRNA1: N=6; shRNA2: N=5; shRNA3: N=4). Data are presented as average of all replicates ± standard error of the mean (paired Student *t* test, $^{**}P<0.01$.). (C-E) Flow cytometric analysis of control shRNA and *HES6* shRNA-transduced CD34⁺Lin⁻BFP⁺ hematopoietic stem and progenitor cells cultured *in vitro* in T-lineage supporting conditions for a total of 3 weeks (N=5), showing gating of differentiation stages based on CD34 and CD7 expression within BFP⁺ cells at day 14 (C), CD7 and CD5 within BFP⁺ cells at day 21 (D) and CD4 and CD8b within BFP⁺ HLA-DR⁻ cells at day 21 (E), with frequencies of BFP⁺ cells of populations of interest. The contour plots shown are representative of five replicates. DN: double negative (CD4⁻CD8⁻); DP: double positive (CD4⁺CD8⁺); SP: single positive.

assay.[28] The frequencies and absolute numbers of BFP⁺ cells were significantly reduced for *HES6* knockdown compared to the control, and the effect increased progressively from day 7 to day 21 (Figure 2D, *Online Supplementary Figure S3B*). We used intracellular (ic) staining of CD179b (λ5 which is part of the pre-B-cell receptor) to investigate the effect of *HES6* knockdown on early B-cell development. Because the fixation and permeabilization process degrades BFP, we were unable to select BFP⁺ cells for flow cytometric analysis, possibly diluting the effect of *HES6* knockdown because non-targeted BFP⁻ cells were included in the analysis. At day 7, we observed no difference in the frequencies of pro-B-cells (CD34⁺icCD179b⁺) in *HES6* knockdown samples compared to the control, although the absolute cell numbers were slightly reduced for two of the three knockdown constructs (*Online Supplementary Figure S3C, D*). At day 21, no clear effect of *HES6* knockdown on the frequencies of pro-B-cells was observed (icCD179b⁺CD19⁻), but there was a significant reduction in the frequencies of pre-B-cells (icCD179b⁺CD19⁺) (Figure 2E, *Online Supplementary Figure S3E*). For both stages, the absolute cell numbers were reduced after *HES6* knockdown

(*Online Supplementary Figure S3F*). Cell surface staining at day 21 revealed that, within the BFP⁺ compartment, the frequencies and absolute numbers of B cells (CD19⁺) were strongly reduced upon *HES6* knockdown, while this was not the case in the BFP⁻ compartment (Figure 2F, *Online Supplementary Figure S3G, H*). Thus, *HES6* is specifically expressed in proliferating large pre-B-cells that received pre-B-cell receptor signaling following successful heavy chain rearrangement, and knockdown of *HES6* inhibits the *in vitro* development of B cells beyond this stage.

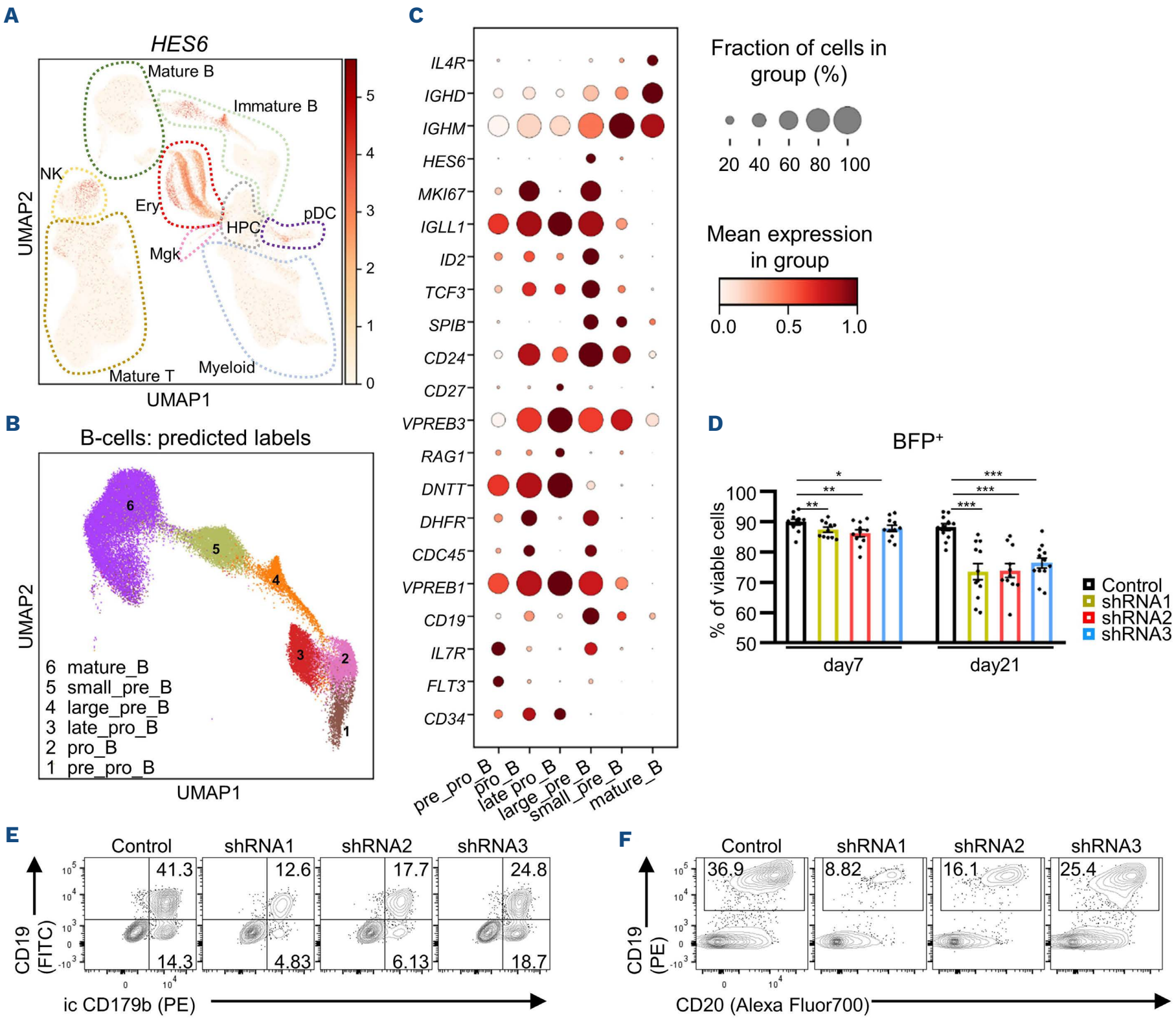

Figure 2. *HES6* is important for early *in vitro* B-cell development. (A, B) UMAP visualization of *HES6* mRNA expression in human bone marrow cells based on single-cell RNA-sequencing data[29] (A) and annotation of stages of B-cell development within the human bone marrow following label transfer (B).[30] (C) Dot plots showing pseudo-bulk log-normalized expression level (color of dots) and percentage of cells within a cluster expressing (size of dots) different genes in clusters of human bone marrow B-lineage cells, as annotated in (B). (D-F) Bar graphs and flow cytometric analysis of control short hairpin (sh)RNA and *HES6* shRNA-transduced CD34⁺Lin⁻BFP⁺ hematopoietic stem and progenitor cells cultured *in vitro* in B-lineage differentiation conditions for a total of 3 weeks. (D) Bar graph showing frequencies of CD45⁺BFP⁺ cells within the population of live cells at day 7 (N=11) and day 21 (N=12; shRNA2: N=11). Data are presented as the average of all replicates ± standard error of the mean (Wilcoxon matched-pairs signed-rank test, **P*<0.05, ***P*<0.01; ****P*<0.001). (E) Flow cytometric analysis of CD19 and intracellular CD179b expression with frequencies of populations of interest within live cells at day 21 (N=7; shRNA2: N=6) and (F) expression of CD19 and CD20 with frequencies of populations of interest within CD45⁺BFP⁺ cells at day 21 (N=12; shRNA2: N=11). Contour plots with frequencies shown are from a representative replicate. UMAP: uniform manifold approximation and projection; NK: natural killer; Ery: erythrocytes; Mgk: megakaryocytes; HPC: hematopoietic precursor cells; pDC: plasmacytoid dendritic cells.

Knockdown of *HES6* impairs plasmacytoid dendritic cell differentiation

We detected *HES6* expression in pDC (Figure 2A) and high expression of this gene is associated with a hematologic malignancy from this lineage (blastic plasmacytoid dendritic cell neoplasm).[19,20] Using an *in vitro* differentiation protocol,[31] we investigated whether HES6 is essential for pDC development. After 14 days of culture, the frequency of BFP$^+$ cells was significantly reduced in *HES6* knockdown cultures compared to the control (*Online Supplementary Figure S4A*). Total cell numbers of BFP$^+$ cells were also reduced for two of the three *HES6* knockdown conditions (*Online Supplementary Figure S4A*). *HES6* knockdown resulted in a significant reduction in the frequency of pDC (CD45RA$^+$CD123$^+$) (Figure 3A, B). For two HES6 targeting shRNA, this was accompanied by a significant reduction in the absolute number of pDC (Figure 3B). Within the BFP$^-$ population, no differences in pDC development were observed between the different conditions, indicating that the observed effect of *HES6* knockdown on pDC development is specific (*Online Supplementary Figure S4B*). Conventional dendritic cells (cDC: CD4$^+$HLA-DR$^+$ non-pDC), which express higher levels of HLA-DR compared to pDC, also developed in these cultures (Figure 3C, *Online Supplementary Figure S4C*). Within the BFP$^+$ compartment, *HES6* knockdown had no influence on the frequencies of cDC compared to that of the control condition, but the absolute cell numbers were reduced for two *HES6*-targeting shRNA (Figure 3D). We also investigated the role of HES6 during NK cell, monocyte and granulocyte development in specific *in vitro* differentiation cultures[28] but knockdown of *HES6* had no consistent impact on the development of these lineages (*Online Supplementary Figure S5*). Overall, these results suggest a functional role for HES6 in pDC development, consistent with its expression in bone marrow pDC.

HES6 is essential for the colony-forming capacity of early hematopoietic precursors

Since our differentiation cultures consistently revealed reduced cellular output upon *HES6* knockdown, we investigated whether this was correlated with a reduced proliferative capacity of the *HES6* knockdown precursors. Gene expression datasets[32] revealed *HES6* expression in multipotent progenitors (MPP), common myeloid progenitors (CMP) and megakaryocyte-erythroid progenitors (MEP) (Figure 4A). To study whether HES6 is important for precursor expansion, we performed colony-forming assays. After 10 days, there was a strong reduction in the number of colonies in *HES6* knockdown conditions compared to the control (Figure 4B) for both myeloid and, in particular, erythroid colonies (Figure 4C). Furthermore, the average cell number per colony was reduced upon *HES6* knockdown compared to the control (Figure 4B). Flow cytometric analysis of these cultures showed large variation in the frequencies of BFP$^+$ cells and, combined with the cellular output of these assays, the total numbers of BFP$^+$ cells were reduced upon *HES6* knockdown (*Online Supplementary Figure S6A*). Within the BFP$^+$ compartment, *HES6* knockdown did not consistently affect CD15$^+$ myeloid cell development but, in contrast, a strong reduction in both the frequencies and absolute numbers of CD235$^+$ erythroblasts was observed (*Online Supplementary Figure S6B-D*), which was confirmed by microscopic analysis (Figure 4D). While there were mainly cells with basophilic cytoplasm in the control samples, which matches the different stages of differentiation along the erythroid lineage that are observed in the bone marrow of healthy individuals, the cytoplasm of the *HES6* knockdown cells was less basophilic, indicating reduced development along the erythroid lineage. Furthermore, we observed fewer mitoses in *HES6* knockdown conditions compared to the control. No differences were observed in the BFP$^-$ cells (*Online Supplementary Figure S6E*). These findings suggest that HES6 is important for the proliferative potential of hematopoietic precursor cells, as well as for their differentiation towards erythroid cells.

HES6 is required for erythroid and megakaryocyte development

We further explored the role of HES6 during erythroid and megakaryocyte development by culturing shRNA-transduced HSPC in liquid culture conditions that induce differentiation towards both lineages. After 5 days, both the frequencies and absolute numbers of BFP$^+$ cells were reduced in the *HES6* knockdown conditions compared to the control (*Online Supplementary Figure S7A, B*). Within the BFP$^+$ population, significant reductions in the frequencies of megakaryocytes (CD41$^+$) and differentiating erythroblasts (characterized by upregulation of CD71 and CD235a) were observed in all three *HES6* knockdown conditions, resulting in significantly reduced cell numbers of these populations (Figure 4E, F, *Online Supplementary Figure S7C, D*). Erythroblasts from *HES6* shRNA-transduced precursors also displayed reduced CD71 expression compared to the control-transduced erythroid cells (*Online Supplementary Figure S7E*). After 10 and 14 days of culture, reductions in the frequencies of BFP$^+$ cells occurred in the control due to loss of BFP expression in differentiating CD235$^+$ erythroblasts, presumably a result of enucleation (*Online Supplementary Figure S7F, G*). Therefore, at days 10 and 14, we used the total population of viable cells for comparison. At day 10, frequencies and total numbers of CD235$^+$ erythroblasts were reduced upon *HES6* knockdown (*Online Supplementary Figure S7H-I*). At day 14, however, no difference could be observed between *HES6* knockdown and control for the frequencies of CD235$^+$ erythroblasts, which probably results from the inclusion of BFP$^-$ cells that mask the effect of *HES6* knockdown in the BFP$^+$ cells, but the absolute cell numbers were still reduced for two of the three *HES6* knockdown constructs (*Online Supplementary Figure S7H, I*). Overall, these results show that HES6 is

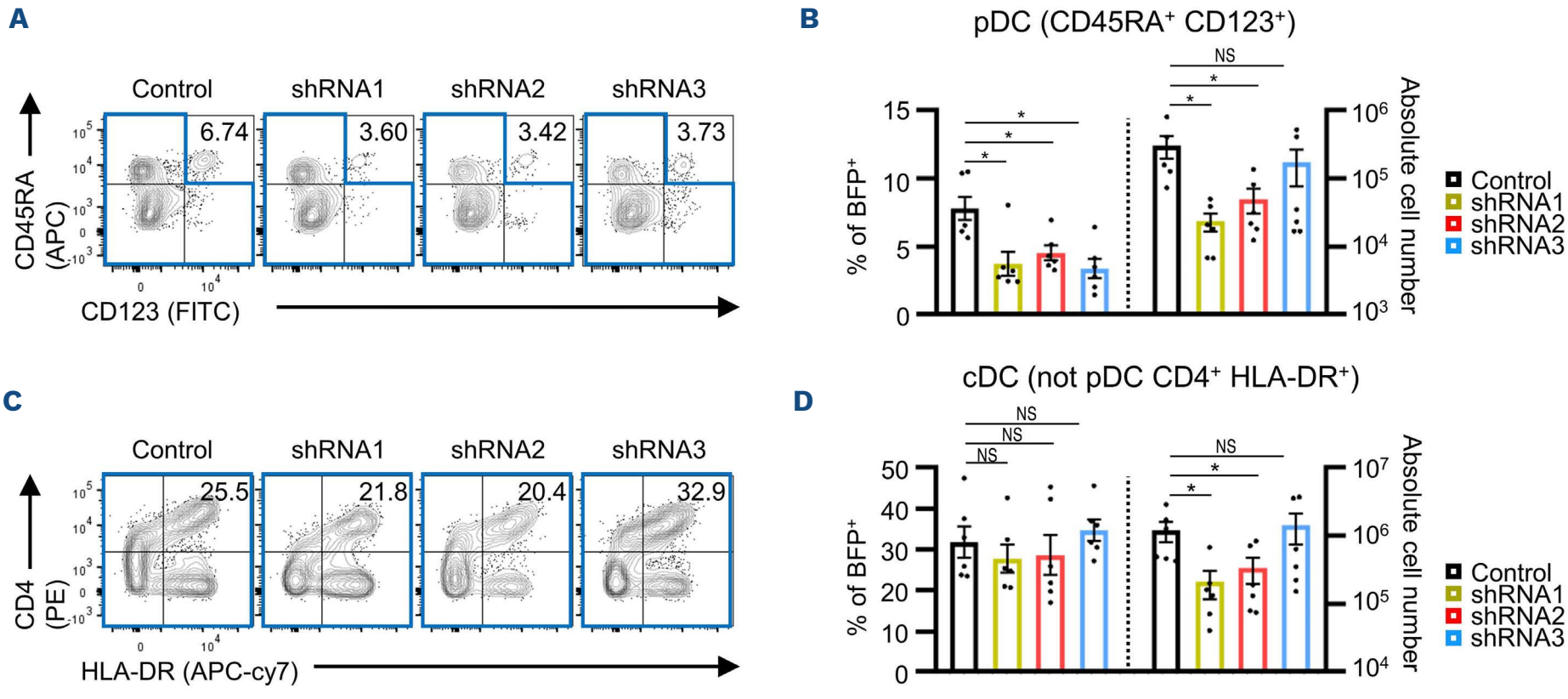

Figure 3. *HES6* knockdown impairs *in vitro* plasmacytoid dendritic cell development. (A, C) Flow cytometric analysis and (B, D) bar graphs of control short hairpin (sh)RNA and *HES6* shRNA-transduced CD34$^+$Lin$^-$BFP$^+$ hematopoietic stem and progenitor cells cultured in dendritic cell (DC) lineage-supporting conditions for a total of 2 weeks (N=6). The contour plots shown are representative of six replicates. (A, C) Flow cytometric analysis showing the development of plasmacytoid DC (pDC: CD45RA$^+$CD123$^+$), gated on BFP$^+$ cells (A), and conventional DC (cDC: CD4$^+$HLA-DR$^+$), gated on BFP$^+$[CD45RA$^+$CD123$^+$]$^-$ cells (C) at day 14. (B, D) Bar graphs showing frequencies and absolute cell numbers of pDC (B) and cDC (D) within the BFP$^+$ population at day 14. Data are presented as the average of all replicates ± standard error of the mean (Wilcoxon matched-pairs signed-rank test, *P<0.05; NS: not significant).

essential for the *in vitro* development of erythrocytes and megakaryocytes from human HSPC, in agreement with its expression in erythrocytes and MEP.

HES6 is needed for *in vivo* precursor engraftment and differentiation towards plasmacytoid dendritic cells, erythroblasts and B cells

To validate our *in vitro* experiments and to study the role of HES6 on human precursor engraftment and differentiation *in vivo*, we injected bulk control and *HES6* shRNA-transduced HSPC intrahepatically into non-obese diabetic–severe combined immunodeficiency Il2RG$^{-/-}$ (NSG) mice (*Online Supplementary Figure S8A*), a well-established mouse model that supports the development of various human hematopoietic lineages.[33] Injected cells comprised non-transduced BFP$^-$ and transduced BFP$^+$ HSPC to create a competitive setting. After 8-9 weeks, both bone marrow and liver were engrafted with similar total human cell numbers for all conditions (Figure 5A). Engraftment of the thymus was too low and inconsistent to allow analysis of *in vivo* T-cell development. The frequency of BFP$^+$ cells before injection was similar and above 10% for both *HES6* knockdown and control-transduced cells, and remained stable in the CD45$^+$ control shRNA-transduced precursors in the bone marrow and liver (Figure 5B). In contrast, there was a clear reduction in BFP$^+$ cells at both anatomical sites for both *HES6* knockdown conditions at the end of the experiment (Figure 5B), indicating that HSPC with reduced *HES6* expression have lower repopulating potential in this competitive setting. A BFP$^+$ population was detected in only six out of 12 *HES6* knockdown animals, thereby limiting the number of datapoints for analysis (Figure 5B). Within the bone marrow of the animals with detectable reconstitution, *HES6* knockdown increased the frequency of NK cells and had no consistent impact on myeloid cells, but both NK and myeloid cell numbers were not significantly altered compared to the control (Figure 5C, D). Although this mouse model is not optimal for supporting human erythroid and megakaryocytic development,[34] we did observe, within the mice that showed reconstitution, a reduction in both megakaryocytes and erythroblasts upon *HES6* knockdown (Figure 5C, D). In addition, the frequency and total number of B cells were decreased in case of *HES6* knockdown (Figure 5C, D). Within the BFP$^+$ population in the liver, both the frequencies and total numbers of pDC were reduced for *HES6* knockdown compared to the control, while the effect on cDC was variable (Figure 5E, F). Overall, and consistent with the *in vitro* colony formation assay, loss of *HES6* expression in human HSPC restricts these cells' *in vivo* repopulating potential. Furthermore, in the mice that showed reconstitution, the pDC, megakaryo-erythroid and B-cell development was reduced upon *HES6* knockdown, in agreement with the findings of the *in vitro* experiments.

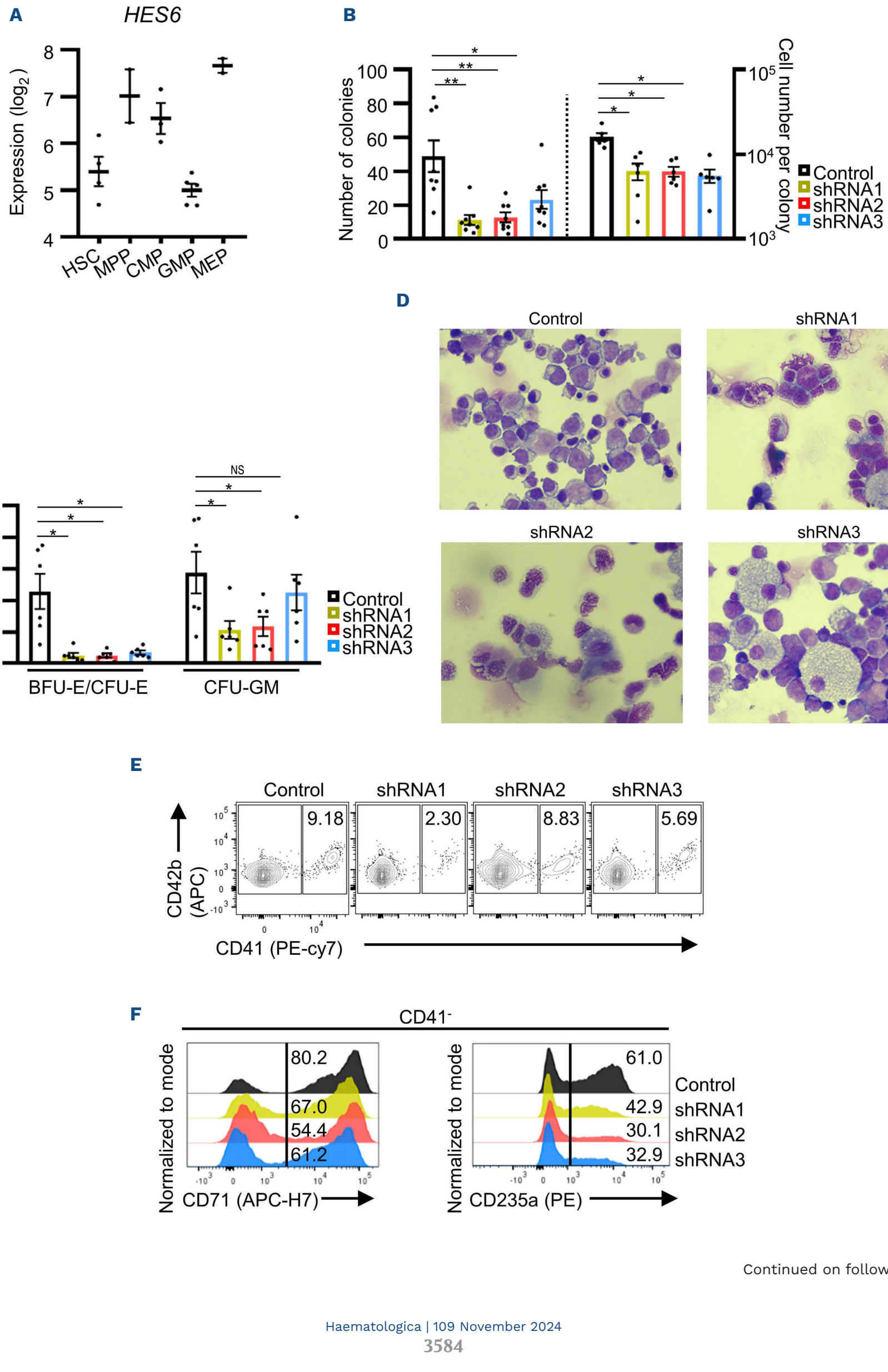

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Figure 4. *HES6* knockdown impairs colony-forming capacity of hematopoietic stem and progenitor cells and *in vitro* erythroid development. (A) Graph derived from published micro-array data[32] showing $\log_2$ normalized probe intensities for *HES6* in sorted subsets of hematopoietic stem and progenitor cells (HSPC) from human bone marrow. The mean is shown with error bars representing standard error of the mean (hematopoietic stem cells: N=4; multipotent progenitors: N=2; common myeloid progenitors: N=3; granulocyte-monocyte progenitors: N=5; megakaryocyte-erythroid progenitors: N=2). (B-D) Analysis of control short hairpin (sh)RNA and *HES6* shRNA-transduced $CD34^+Lin^-BFP^+$ HSPC cultured in a semi-solid colony-forming assay, Methocult, for a total of 10 days. Bar graphs showing number of total colonies (B, left; N=8), absolute cell numbers per colony (B, right; N=6) and number of erythroid (BFU-E/CFU-E) or myeloid (CFU-GM) colonies (C) (N=8). (B, C) Data are presented as the average of all replicates ± standard error of the mean (Wilcoxon matched-pairs signed-rank test, **P*<0.05, ***P*<0.01; NS: not significant). (D) Representative microscopic images of cytospin slides using a Leica DM 3000 LED microscope (original magnification, 500x). (E, F) Flow cytometric analysis of control shRNA and *HES6* shRNA-transduced $CD34^+Lin^-BFP^+$ HSPC cultured in megakaryocyte/erythroid-lineage supporting conditions for a total of 2 weeks, showing gating and frequencies of megakaryocytes ($CD41^+$) and erythroblasts ($CD71^+$ erythroblasts and late-stage $CD235^+$ erythroblasts) in the $CD45^+BFP^+$ population at day 5 (N=14, shRNA3: N=11). The contour plots (E) and histograms (F) shown are representative of all replicates. HSC: hematopoietic stem cells; MPP: multipotent progenitors; CMP: common myeloid progenitors; GMP: granulocyte-monocyte progenitors; MEP: megakaryocyte-erythroid progenitors; BFU-E: burst-forming unit-erythroid; CFU-E: colony-forming unit-erythroid; CFU-GM: colony-forming unit - granulocyte-monocyte.

HES6 knockdown affects gene expression during erythroid and megakaryocyte development

To study the downstream changes following *HES6* knockdown, we performed RNA sequencing on sorted subpopulations from control and *HES6* shRNA-transduced HSPC after 4 days in liquid cultures that induce erythroid and megakaryocyte differentiation (Figure 6A, *Online Supplementary Figure S9A*). In control samples, megakaryocytic genes (*VWF*, *GP1BA*, *GP9*, *ITGA2B*) were specifically expressed in megakaryocytes ($CD41^+$) while erythroid-specific genes (*EPOR*, *TFRC*, *E2F4*, *E2F8*) were induced in $CD71^+CD235^-$ early and $CD71^+CD235^+$ late erythroblasts, validating our sorting strategy (Figure 6B). *HES6* and *TCF3* expression was highest in erythroid cells, while *HES1* showed the opposite pattern with highest expression in megakaryocytes. While megakaryocytes express both *GATA1* and *GATA2*, late erythroblasts only express *GATA1*. Consistent with the highest expression of *HES6*, the impact of *HES6* knockdown on gene expression was most pronounced in erythroblasts, but also notable in megakaryocytes in which we observed biological variation for one donor (*Online Supplementary Figure S9B*). In $CD34^-$ cells, the biological variation between donors dominated over the effect of *HES6* knockdown (*Online Supplementary Figure S9B*). Differential gene expression analysis revealed more differentially expressed genes following *HES6* knockdown in erythroblasts than in megakaryocytes or $CD34^-$ cells, with substantial overlap in erythroblast stages (*Online Supplementary Figure S9C*). Within these, we observed significantly reduced expression of erythroid-associated genes (*TFRC* which encodes *CD71*, *HBB*, *EPCAM*, *E2F4*) (Figure 6C, D) and, consistently, gene set enrichment analysis revealed enriched erythroblast-related gene sets in control-transduced samples (*Online Supplementary Figure S9D*). Compared to the control, *HES6* knockdown erythroid cells failed to downregulate *GATA2* and *ID2* expression (Figure 6B-D). No impact on *GATA1* expression was observed (*data not shown*). Differential gene expression analysis within megakaryocytes showed significantly reduced expression of genes important for megakaryocyte development (*CD36*, *DLK1*) and proliferation (*MKI67*, *HEMGN*) (Figure 6E). Within the more heterogenous and more immature $CD34^-$ population, pDC-associated genes (*IRF8*, *TCF4*),[35,36] B-lineage genes (*BLNK*, *IGLL1*, *IGHM*),[37,38] the E-protein encoding gene *TCF3* and *RUNX2*, important for hematopoietic precursor cells and pDC,[39-41] were all downregulated in *HES6* knockdown cells compared to the control (Figure 6F).

Given that the GATA2 to GATA1 switch is critical to permit erythroid development,[42] we investigated whether failure to downregulate GATA2 mediated the block in erythroid development upon *HES6* knockdown. To do this, we cultured *HES6-GATA2* double-knockdown HSPC in erythroid culture conditions (*Online Supplementary Figure S10A*). Both *GATA2* shRNA reduced *GATA2* expression by 50% (Figure 7A) and this did not significantly change megakaryocyte development compared to *HES6* knockdown alone (Figure 7B, *Online Supplementary Figure S10B*), although a trend towards an increased frequency of megakaryocytes was observed. Compared to the effect of *HES6* knockdown alone, knockdown of both genes further decreased erythroid development, in terms of both frequencies and total cell numbers (Figure 7B, *Online Supplementary Figure S10B*). Single *GATA2* knockdown had a similar effect on megakaryocyte and erythroid development (Figure 7C, *Online Supplementary Figure S10C*). Analysis of chromatin immunoprecipitation-sequencing data[23] revealed binding of HES6 at genes associated with erythroid (*HBB*, *TFRC*) and megakaryocyte (*HEMGN*, *CD36*) differentiation and proliferation (*MKI67*) (Figure 8A, *Online Supplementary Figure S11A*), which were downregulated upon *HES6* knockdown (Figure 6C-E). Furthermore, we found HES6 binding peaks in genes important for T- and B-cell development (*BLNK*, *IGHM*, *TCF3*, *TCF12*) and pDC development (*TCF4*, *RUNX2*) (*Online Supplementary Figure S11A*). For all these HES6-bound target genes, only *HBB* and *IGHM* showed overlapping GATA1 binding[23] (*data not shown*). These data indicate that HES6 has an essential role during erythroid development which is independent of *GATA2* expression and suggest that HES6 may directly regulate the expression of genes that are important for erythroid, megakaryocyte, pDC, B- and T-cell development.

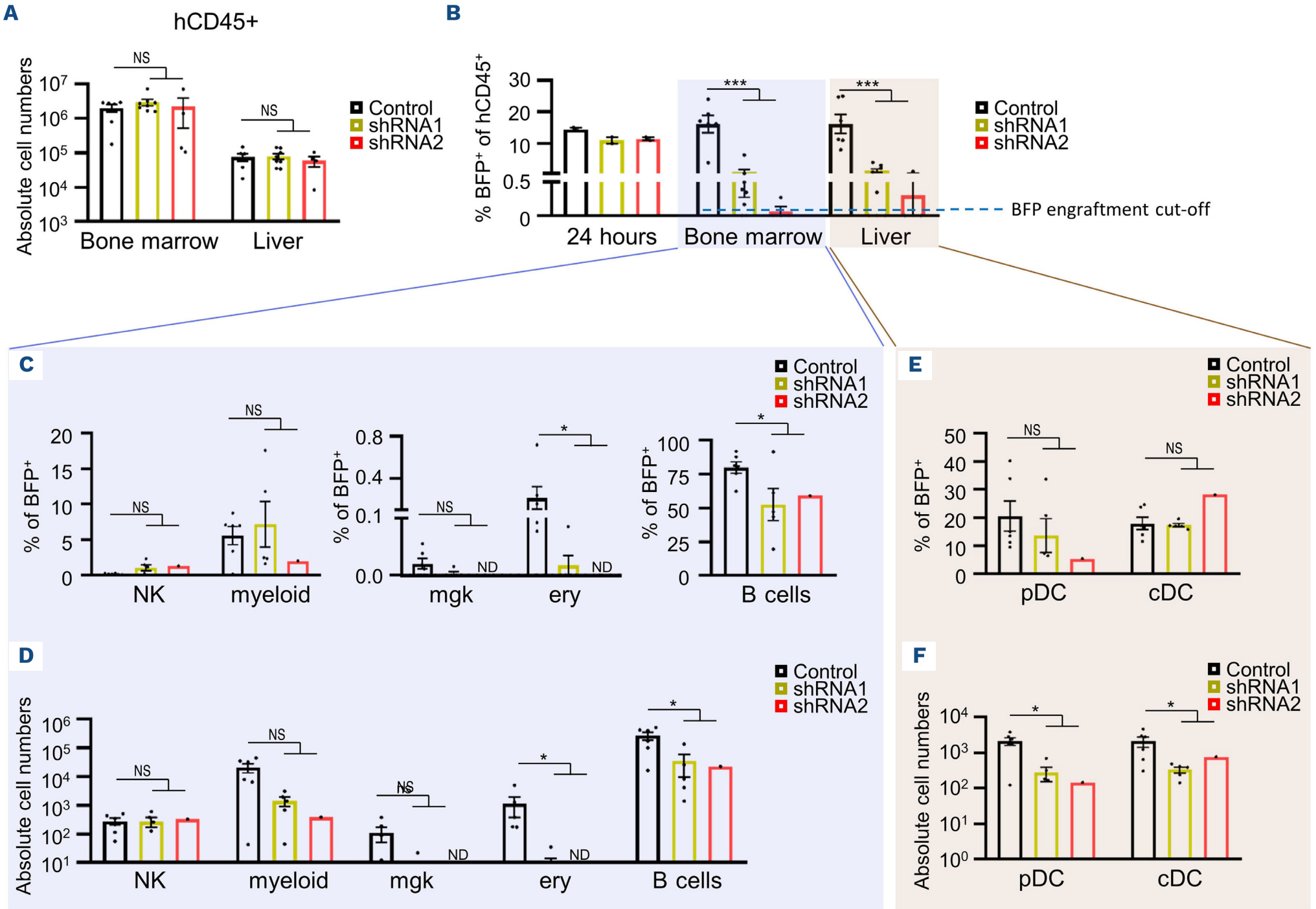

Figure 5. Knockdown of *HES6* reduces the *in vivo* engraftment potential of hematopoietic stem and progenitor cells and their *in vivo* capacity to differentiate into megakaryocytes, erythroid, B and plasmacytoid dendritic cells. (A-F) Bar graphs showing the *in vivo* engraftment of hCD45$^+$ cells (A, B, control N=6, shRNA1 N=8, shRNA2 N=4) and the specific lineage differentiation within the BFP$^+$ population of engrafted mice (C-F, control N=6, shRNA1 N=5, shRNA2 N=1). (A) Bar graph showing absolute hCD45$^+$ cell numbers in the bone marrow and liver for control and *HES6* knockdown conditions. (B) Bar graphs showing frequencies of BFP$^+$ cells within the hCD45$^+$ population at the start of the experiment after 24 hours of transduction (left), at the end of the experiment in the bone marrow (center) and liver (right) and the cutoff for BFP engraftment, resulting in fewer datapoints in subsequent analysis of the BFP$^+$ population within engrafted mice (of which results are shown in panels C-F). (C, D) Bar graphs showing frequencies (C) and absolute cell numbers (D) of subpopulations within BFP$^+$ populations in the bone marrow. NK: CD56$^+$CD3$^-$, myeloid: CD33$^+$, mgk: CD41$^+$, ery: CD71$^+$CD41$^-$, B cells: CD19$^+$. (E, F) Bar graphs showing frequencies (E) and absolute cell numbers (F) of subpopulations within BFP$^+$ populations in the liver. pDC: CD123$^+$CD45RA$^+$, cDC: not pDC CD4$^+$HLA-DR$^+$. Data are presented as the average of all replicates ± standard error of the mean (Mann Whitney U test on control *vs.* *HES6* knockdown; pooled shRNA1 and shRNA2). *P<0.05, ***P<0.001; NS: not significant. ND: not detected; NK: natural killer cells; mgk: megakaryocytes; ery: erythroblasts; pDC: plasmacytoid dendritic cells; cDC: conventional dendritic cells.

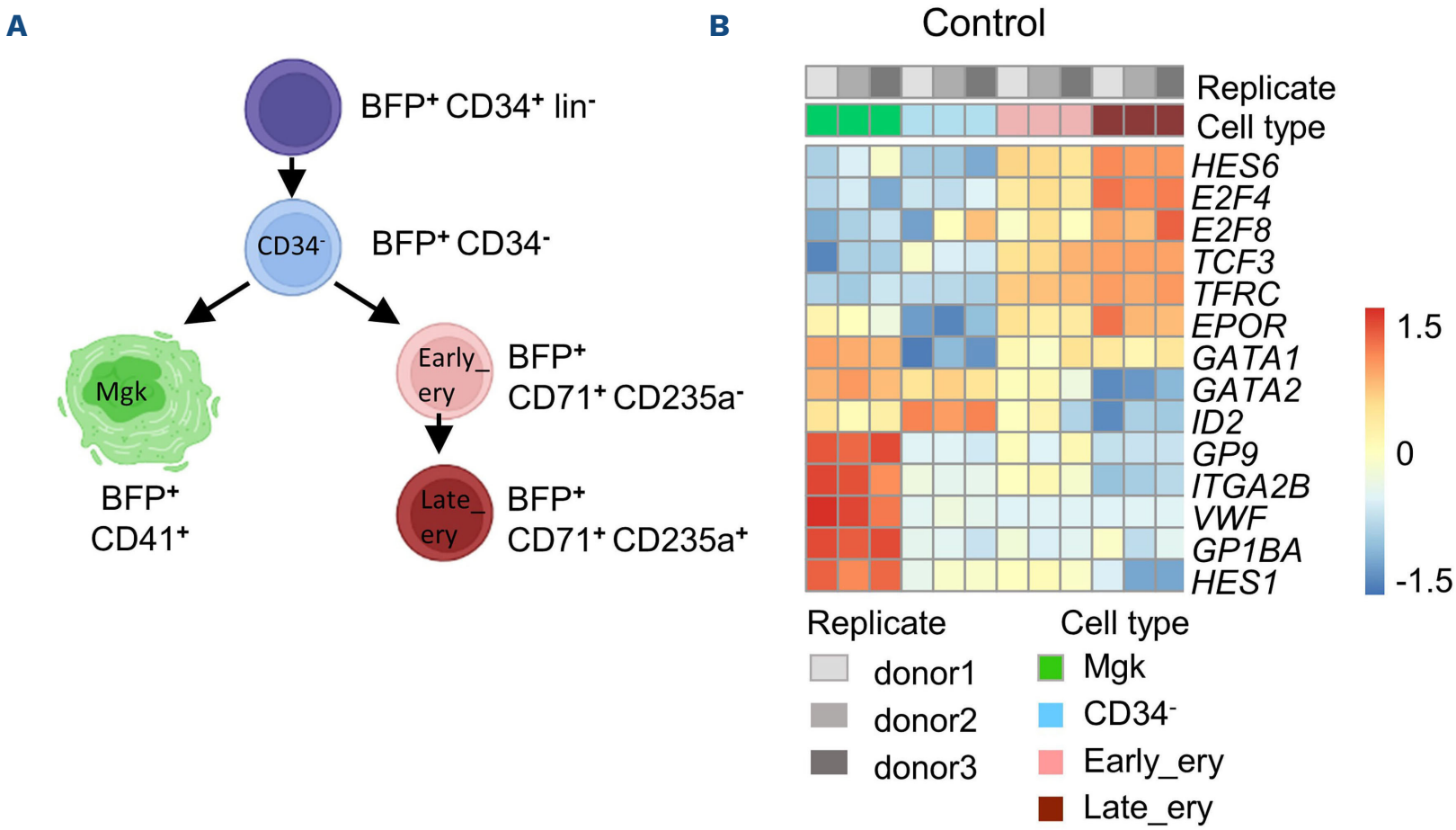

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Figure 6. RNA-sequencing analysis reveals molecular pathways downstream of *HES6* knockdown during erythroid development. (A) Overview of development of control short hairpin (sh)RNA and *HES6* shRNA-transduced (shRNA1 and shRNA2 were used) $CD34^+Lin^-BFP^+$ hematopoietic stem and progenitor cells cultured for 4 days in megakaryocyte/erythroid-lineage-supporting conditions. Bulk RNA sequencing was performed on the four indicated subpopulations. (B, C) Results from bulk RNA-sequencing data from three donors on the four subpopulations as shown in Figure 6A and *Online Supplementary Figure S9A*. (B) Heatmap showing expression of genes in different cell populations in control samples. (C-F) Volcano plots showing differentially up- and down-regulated genes (blue and red, respectively) in different cell populations. (E, F) In the volcano plots of megakaryocytes and $CD34^-$ precursors, *HES6* is indicated in gray because its expression was not significantly downregulated. (F) In the volcano plot of $CD34^-$ precursors, *TCF3* is indicated in gray considering downregulation was significant (*P* adjusted <0.05) but $\log_2$ fold-change values were just below the cut-off. Mgk: megakaryocytes; ery: erythroblasts; *P*adj: adjusted *P* value.

HES6 facilitates proliferation by mediating the G1-to-S phase transition

Since *HES6* knockdown reduced cellular output and downregulated genes associated with proliferation, we investigated the effect of *HES6* knockdown on proliferation. Gene set enrichment analysis of late erythroblast samples as well as megakaryocytes showed enrichment of cell cycle related gene sets in the control, including MYC targets, genes related to G1-S specific transcription, G2M checkpoint genes and E2F targets (Figure 8B, *Online Supplementary Figure S11B*). Interestingly, *TFRC*, *E2F4* and *E2F8* were shown to be important for cell proliferation[43-45] and expressed during erythroid development (Figure 6B) but downregulated upon *HES6* knockdown in late erythroblasts (Figure 6D). This was confirmed for *TFRC* at the protein level (*Online Supplementary Figure S7E*). Furthermore, in late erythroblasts, genes with specific *E2F4*-binding motifs were enriched in control samples (*Online Supplementary Figure S11C*), although the difference was not statistically significant. To investigate whether *HES6* knockdown had an effect on cell proliferation or survival during erythroid development, we performed a 5-ethynyl-2'-deoxyuridine (EdU) incorporation and apoptosis assay. A significant decrease in the frequency of cells in the proliferating S phase (EdU^+) and a significant increase in cells in the resting G1 phase (EdU^- and 2N DNA content) were observed upon *HES6* knockdown compared to in control-transduced cells (Figure 8C, *Online Supplementary Figure S11D*). Given that we did not detect any consistent differences in the relative number of early or late apoptotic cells upon *HES6* knockdown compared to control (*Online Supplementary Figure S11E-F*), these results indicate that HES6 is required for the proliferation of erythroid cells and that this might involve the regulation of genes that mediate cell cycle progression.

Discussion

While important functions for several HES gene family members had been revealed in both normal and malignant hematopoiesis,[5,6,9] only very recently has a first role for HES6 been demonstrated as a GATA1 cofactor during

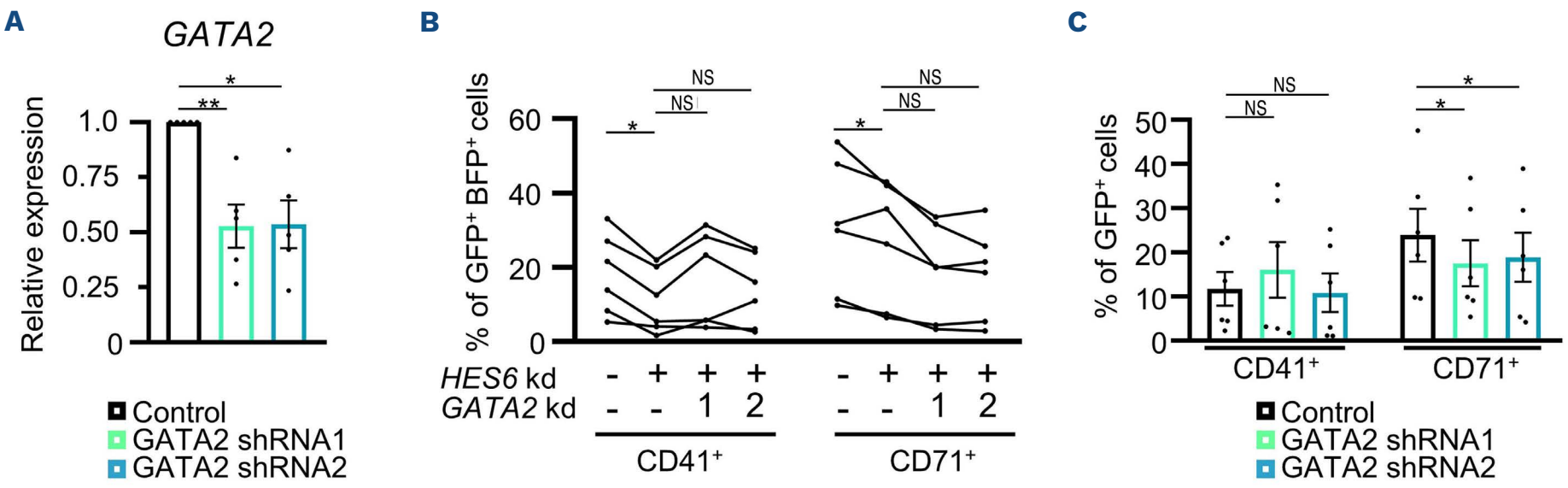

Figure 7. *GATA2* knockdown fails to rescue impaired *in vitro* megakaryo-erythroid development upon *HES6* knockdown. (A-C) Bar graphs and line graphs showing results of *in vitro* experiments to study the impact of double *GATA2-HES6* knockdown (GFP^+BFP^+) (B) or single *GATA2* knockdown (GFP^+BFP^-) (A, C) on human megakaryo-erythrocyte development (N=6) as described in *Online Supplementary Figure S10A*. (A) Quantitative real-time polymerase chain reaction analysis showing *GATA2* expression in cord blood $CD34^+Lin^-GFP^+BFP^-$ cells transduced with a control short hairpin (sh)RNA or two different *GATA2* shRNA, normalized to expression of the housekeeping gene *SDHA* and relative to the expression in the control shRNA condition. Data are presented as the average of five replicates ± standard error of the mean (SEM) (paired Student *t* test). (B, C) Line graphs and bar graphs showing megakaryocyte ($CD41^+$) and erythroblast ($CD71^+$) percentages within double *GATA2-HES6* knockdown (B) or single *GATA2* knockdown (C) cultures (N=6) with populations gated similarly as shown in Figure 4E, F. (B, C) Data are presented as the average of six replicates and error bars indicate the SEM (Wilcoxon matched-pairs signed-rank test, **P*<0.05, ***P*<0.01, NS: not significant).

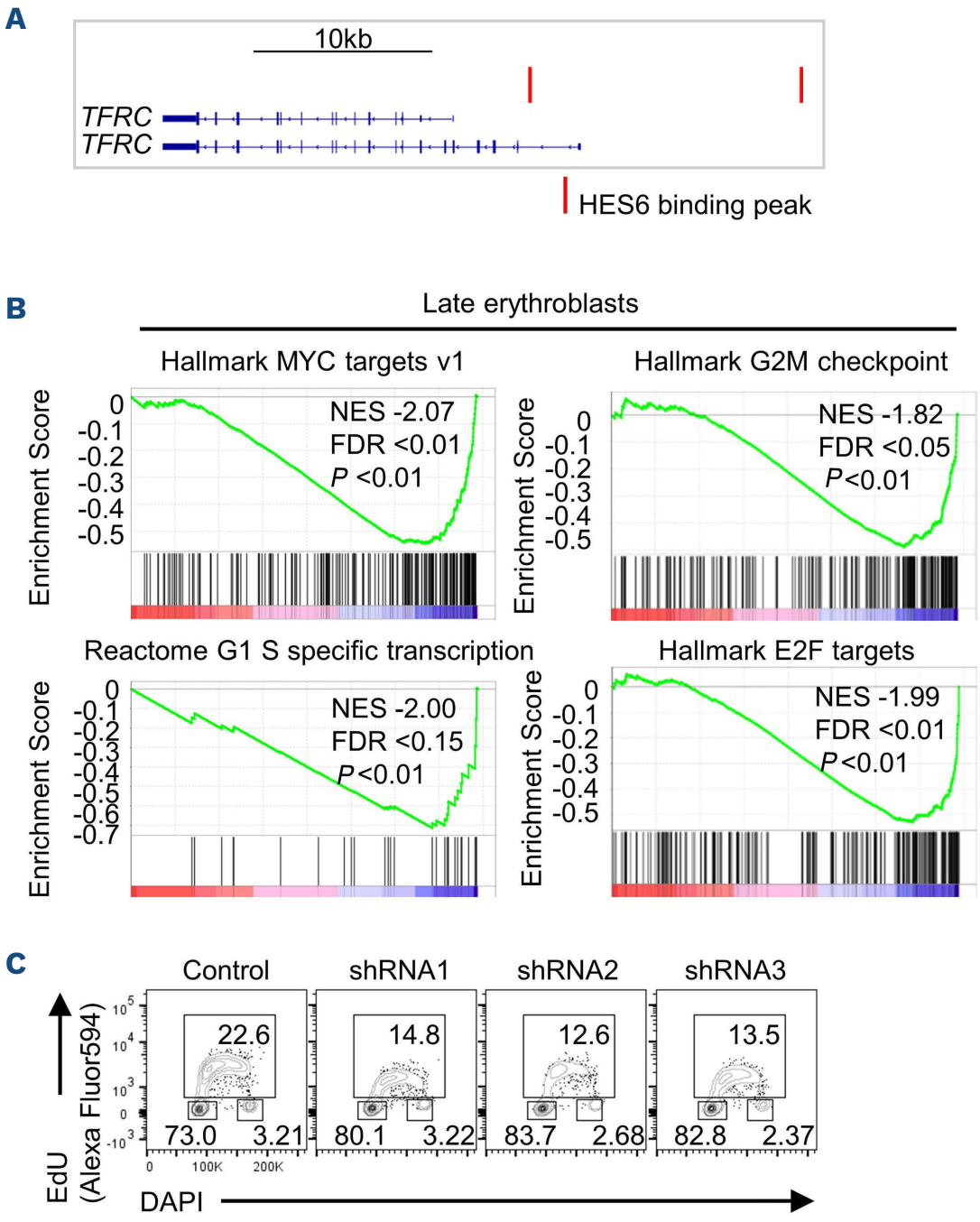

Figure 8. *HES6* knockdown impacts cell cycle progression. (A) Analysis of recently published chromatin immunoprecipitation-sequencing data of *HES6* in erythroid cells;[23] significant *HES6* binding peaks for the *TFRC* gene are shown in red. (B) Results from data analysis of bulk RNA sequencing of sorted cell populations as discussed in Figure 6. Pre-ranked gene set enrichment analysis within late erythroblasts shows enrichment of cell-cycle related gene sets in the control sample. (C) Results of control short hairpin (sh)RNA and *HES6* shRNA-transduced $CD34^+Lin^-BFP^+$ hematopoietic stem and progenitor cells cultured in megakaryocyte/erythroid-lineage-supporting conditions for 3 days (N=5), showing gating and frequencies of cell-cycle stages within viable cells for a representative replicate (G1: EdU^- DAPI low [DNA 2N]; S: EdU^+; G2M: EdU^- DAPI high [DNA 4N]). NES: normalized enrichment score; FDR: false detection rate; EdU: 5-ethynyl-2'-deoxyuridine; DAPI: 4',6-diamidino-2-phenylindole.

erythropoiesis.[23] In this study, we explored *HES6* expression and function during human hematopoiesis *in vivo* and *in vitro* and provide insights into potential lineage- and stage-specific roles for this transcription factor in human hematopoiesis.

Using well-described *in vitro* differentiation culturing systems[28,31] we showed a clear impact of *HES6* knockdown on the development of megakaryocytes, erythrocytes, pDC, B cells and T cells, consistent with its expression in these lineages. While this study confirms the recently reported importance of HES6 in normal erythropoiesis,[23] our work reveals other mechanisms through which HES6 mediates this developmental process. In contrast to Wang *et al.*,[23] we did not observe GATA1 binding at most HES6 target genes that we identified in our RNA-sequencing data, suggesting GATA1-independent functions for HES6. Consistently, *GATA1* expression was not reduced following *HES6* knockdown in erythroblasts. Our knockdown experiments resulted in significantly fewer differentially expressed genes compared to those found by Wang *et al.*,[23] which presumably results from different experimental approaches. Our differential gene expression analysis is based on shared differences induced by two different *HES6* shRNA, compared to a single shRNA in their study. Moreover, we sorted surface marker-defined subpopulations prior to sequencing, allowing a comparison of developmental stage-matched cells and excluding an impact of different differentiation stages on gene expression. We could not confirm a higher degree of apoptosis upon *HES6* knockdown,[23] but instead show that HES6 regulates proliferation by mediating the G- to S-cell cycle phase transition. RNA-sequencing and chromatin immunoprecipitation-sequencing[23] data suggest direct regulatory roles for HES6 in positively regulating *TFRC* (encoding the transferrin receptor CD71) and *MKI67* expression, two important mediators of proliferation that do not display GATA1 binding. We confirmed reduced *TFRC* expression following *HES6* knockdown at the protein (CD71) level. Interestingly, previous work in our laboratory showed that TFRC is specifically upregulated following pre-T-cell receptor signaling at the $CD4^+CD28^+CD3^-$ stage of human T-cell development,[46] the stage at which *HES6* expression and clonal expansion are induced. We suggest that the

requirement for HES6 is based on its induction of *TFRC* expression and thus the promotion of proliferation after pre-B-cell receptor/T-cell receptor signaling, analogous to the observations in erythropoiesis. Consistently, proliferation and differentiation are tightly coupled during erythropoiesis[47] and T-cell development.[48] Importantly, *GATA2* levels were higher in erythroblasts upon *HES6* knockdown and this perturbed GATA2 to GATA1 switch could potentially have an impact on erythroid development.[42] However, Ahmed *et al.*[49] showed that *GATA2* downregulation during erythroid development is a consequence of shortened cell cycle length and not the result of reduced expression. Therefore, we hypothesize that the increased *GATA2* expression in *HES6* knockdown erythroblasts results from their reduced proliferation, in agreement with all our experimental data that support such a role for HES6 in mediating proliferation. Consistent with this, *GATA2* knockdown did not rescue erythroid development following *HES6* knockdown. The proliferative capacity of HES6 in hematopoietic cells is consistent with its oncogenic role in various types of cancer.[13-17] Although the precise molecular mechanisms are still unclear, a link between HES6 and MYC activity has been proposed,[18] and MYC target genes were also depleted in *HES6* shRNA-transduced erythroblasts. Therefore, our study may help to unravel the oncogenic role of HES6, also in blastic plasmacytoid dendritic cell neoplasm.

Our study also reveals an important functional role for HES6 in HSPC function, consistent with its high expression in MPP, although the mechanism is presently unclear. *HES6* knockdown HSPC displayed a strongly reduced colony-forming capacity *in vitro* and impaired engraftment potential in an *in vivo* competitive transplantation model. This decreased proliferative capacity of *HES6* knockdown HSPC may have also caused the observed reduction in the absolute numbers of monocytes, granulocytes, cDC and NK cells, despite the lack of impact on the frequencies of these cells. We therefore did not connect these findings with a direct role for HES6 in the differentiation of these lineages. In each case, given that the few mice that showed reconstitution with *HES6* shRNA also displayed reduced erythroid, pDC and B-cell development, consistent with our *in vitro* experiments, we believe that our study reveals novel functional roles for HES6 in the engraftment potential of human HSPC and in their differentiation potential towards particular hematopoietic lineages.

Disclosures

No conflicts of interest to disclose.

Contributions

TDV, JP, and TT conceived the study. TDV, FVN, and TT were responsible for the methodology. TDV, LB, TP, NO, YVD, SDM, IV, and MC performed the investigations. BV, JP, and TT acquired resources for the study. TT supervised the study and, together with TDV, wrote the manuscript. All the authors read and agreed to the submitted version of the manuscript.

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Data-sharing statement

The RNA-sequencing data are available through GEO Series accession number GSE229196.

References

1. Rodrigues CP, Shvedunova M, Akhtar A. Epigenetic regulators as the gatekeepers of hematopoiesis. Trends Genet. 2021;37(2):125-142.
2. Wilson NK, Foster SD, Wang X, et al. Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. Cell Stem Cell. 2010;7(4):532-544.
3. Weng AP, Ferrando AA, Lee W, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science. 2004;306(5694):269-271.
4. Klinakis A, Lobry C, Abdel-Wahab O, et al. A novel tumour-suppressor function for the Notch pathway in myeloid leukaemia. Nature. 2011;473(7346):230-233.
5. D'Altri T, Gonzalez J, Aifantis I, Espinosa L, Bigas A. Hes1 expression and CYLD repression are essential events downstream of Notch1 in T-cell leukemia. Cell Cycle. 2011;10(7):1031-1036.
6. Espinosa L, Cathelin S, D'Altri T, et al. The Notch/Hes1 pathway sustains NF-κB activation through CYLD repression in T cell leukemia. Cancer Cell. 2010;18(3):268-281.
7. Varnum-Finney B, Dallas MH, Kato K, Bernstein ID. Notch target Hes5 ensures appropriate Notch induced T- versus B-cell

choices in the thymus. Blood. 2008;111(5):2615-2620.
8. Wendorff AA, Koch U, Wunderlich FT, et al. Hes1 is a critical but context-dependent mediator of canonical Notch signaling in lymphocyte development and transformation. Immunity. 2010;33(5):671-684.
9. De Decker M, Lavaert M, Roels J, et al. HES1 and HES4 have non-redundant roles downstream of Notch during early human T-cell development. Haematologica. 2021;106(1):130-141.
10. Murai K, Vernon AE, Philpott A, Jones P. Hes6 is required for MyoD induction during gastrulation. Dev Biol. 2007;312(1):61-76.
11. Gratton MO, Torban E, Jasmin SB, Theriault FM, German MS, Stifani S. Hes6 promotes cortical neurogenesis and inhibits Hes1 transcription repression activity by multiple mechanisms. Mol Cell Biol. 2003;23(19):6922-6935.
12. Jhas S, Ciura S, Belanger-Jasmin S, et al. Hes6 inhibits astrocyte differentiation and promotes neurogenesis through different mechanisms. J Neurosci. 2006;26(43):11061-11071.
13. Haapa-Paananen S, Kiviluoto S, Waltari M, et al. HES6 gene is selectively overexpressed in glioma and represents an important transcriptional regulator of glioma proliferation. Oncogene. 2012;31(10):1299-1310.
14. Carvalho FLF, Marchionni L, Gupta A, et al. HES6 promotes prostate cancer aggressiveness independently of Notch signalling. J Cell Mol Med. 2015;19(7):1624-1636.
15. Xu Y, Liu X, Zhang H, et al. Overexpression of HES6 has prognostic value and promotes metastasis via the Wnt/ß-catenin signaling pathway in colorectal cancer. Oncol Rep. 2018;40(3):1261-1274.
16. Zhang P, Yang M, Zhang Y, et al. Dissecting the single-cell transcriptome network underlying gastric premalignant lesions and early gastric cancer. Cell Rep. 2020;30(12):4317.
17. Pandiani C, Strub T, Nottet N, et al. Single-cell RNA sequencing reveals intratumoral heterogeneity in primary uveal melanomas and identifies HES6 as a driver of the metastatic disease. Cell Death Differ. 2021;28(6):1990-2000.
18. Krossa I, Strub T, Martel A, et al. Recent advances in understanding the role of HES6 in cancers. Theranostics. 2022;12(9):4374-4385.
19. Dijkman R, Van Doorn R, Szuhai K, Willemze R, Vermeer MH, Tensen CP. Gene-expression profiling and array-based CGH classify CD4+CD56+ hematodermic neoplasm and cutaneous myelomonocytic leukemia as distinct disease entities. Blood. 2007;109(4):1720-1727.
20. Sapienza MR, Fuligni F, Agostinelli C, et al. Molecular profiling of blastic plasmacytoid dendritic cell neoplasm reveals a unique pattern and suggests selective sensitivity to NF-kB pathway inhibition. Leukemia. 2014;28(8):1606-1616.
21. Roy A, Wang G, Iskander D, et al. Transitions in lineage specification and gene regulatory networks in hematopoietic stem/progenitor cells over human development. Cell Rep. 2021;36(11):109698.
22. Da Cunha AF, Brugnerotto AF, Duarte AS, et al. Global gene expression reveals a set of new genes involved in the modification of cells during erythroid differentiation. Cell Prolif. 2010;43(3):297-309.
23. Wang Z, Wang P, Zhang J, et al. The novel GATA1-interacting protein HES6 is an essential transcriptional cofactor for human erythropoiesis. Nucleic Acids Res. 2023;51(10):4774-4790.
24. Roels J, Kuchmiy A, De Decker M, et al. Distinct and temporary-restricted epigenetic mechanisms regulate human αβ and γδ T cell development. Nat Immunol. 2020;21(10):1280-1292.
25. Durinck K, Wallaert A, Van de Walle I, et al. The Notch driven long non-coding RNA repertoire in T-cell acute lymphoblastic leukemia. Haematologica. 2014;99(12):1808-1816.
26. Loontiens S, Dolens AC, Strubbe S, et al. PHF6 expression levels impact human hematopoietic stem cell differentiation. Front Cell Dev Biol. 2020;8:599472.
27. Roels J, Van Hulle J, Lavaert M, et al. Transcriptional dynamics and epigenetic regulation of E and ID protein encoding genes during human T cell development. Front Immunol. 2022;13:960918
28. Dolens AC, Van de Walle I, Taghon T. Approaches to study human T cell development. In: Bosselut R, Vacchio MS, eds. T-Cell Development: Methods and Protocols. Springer New York; 2016. p. 239-251.
29. Regev A, Teichmann SA, Lander ES, et al. The human cell atlas. Elife. 2017;6:1-30.
30. Suo C, Dann E, Goh I, et al. Mapping the developing human immune system across organs. Science. 2022;376(6597):eabo0510.
31. Kirkling ME, Cytlak U, Lau CM, et al. Notch signaling facilitates in vitro generation of cross-presenting classical dendritic cells. Cell Rep. 2018;23(12):3658-3672.
32. Rapin N, Bagger FO, Jendholm J, et al. Comparing cancer vs normal gene expression profiles identifies new disease entities and common transcriptional programs in AML patients. Blood. 2014;123(6):894-904.
33. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2Rγnull mice engrafted with mobilized human hemopoietic stem cells. J Immunol. 2005;174(10):6477-6489.
34. Mende N, Rahmig S, Waskow C. Multilineage readout after HSC expansion – erythrocytes matter. Cell Cycle. 2016;15(8):1032-1033.
35. Kuo YH, Zaidi SK, Gornostaeva S, Komori T, Stein GS, Castilla LH. Runx2 induces acute myeloid leukemia in cooperation with Cbfβ-SMMHC in mice. Blood. 2009;113(14):3323-3332.
36. Sichien D, Scott CL, Martens L, et al. IRF8 transcription factor controls survival and function of terminally differentiated conventional and plasmacytoid dendritic cells, respectively. Immunity. 2016;45(3):626-640.
37. Kwon K, Hutter C, Sun Q, et al. Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. Immunity. 2008;28(6):751-762.
38. Minegishi Y, Rohrer J, Coustan-Smith E, et al. An essential role for BLNK in human B cell development. Science. 1999;286(5446):1954-1957.
39. Cisse B, Caton ML, Lehner M, et al. Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. Cell. 2008;135(1):37-48.
40. Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B. Continuous expression of the transcription factor E2-2 maintains the cell fate of mature plasmacytoid dendritic cells. Immunity. 2010;33(6):905-916.
41. Sawai CM, Sisirak V, Ghosh HS, et al. Transcription factor Runx2 controls the development and migration of plasmacytoid dendritic cells. J Exp Med. 2013;210(11):2151-2159.
42. Bresnick EH, Lee HY, Fujiwara T, Johnson KD, Keles S. GATA switches as developmental drivers. J Biol Chem. 2010;285(41):31087-31093.
43. Kinross KM, Clark AJ, Iazzolino RM, Humbert PO. E2f4 regulates fetal erythropoiesis through the promotion of cellular proliferation. Blood. 2006;108(3):886-895.
44. Ye L, Guo L, He Z, et al. Upregulation of E2F8 promotes cell

proliferation and tumorigenicity in breast cancer by modulating G1/S phase transition. Oncotarget. 2016;7(17):23757-23771.
45. Fouquet G, Thongsa-ad U, Lefevre C, et al. Iron-loaded transferrin potentiates erythropoietin effects on erythroblast proliferation and survival: a novel role through transferrin receptors. Exp Hematol. 2021;99:12-20.
46. Taghon T, Van de Walle I, De Smet G, et al. Notch signaling is required for proliferation but not for differentiation at a well-defined β-selection checkpoint during human T-cell development. Blood. 2009;113(14):3254-3263.
47. Kadri Z, Lefevre C, Goupille O, et al. Erythropoietin and IGF-1 signaling synchronize cell proliferation and maturation during erythropoiesis. Genes 2015;29(24):2603-2616.
48. Kreslavsky T, Gleimer M, Miyazaki M, et al. β-selection-induced proliferation is required for αβ T cell differentiation. Immunity. 2012;37(5):840-853.
49. Ahmed N, Kull T, Loeffler D, Hoppe P, van den Akker E, Schroeder T. Live single cell quantification of GATA switch dynamics during adult and developmental erythropoiesis. Exp Hematol. 2021;12(100):S44.