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METHODS AND REAGENTS

Utilization of the AAVS1 safe harbor locus for hematopoietic specific transgene expression and gene knockdown in human ES cells

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Abstract Human pluripotent stem cells offer a powerful system to study human biology and disease. Here, we report a system to both express transgenes specifically in ES cell derived hematopoietic cells and knockdown gene expression stably throughout the differentiation of ES cells. We characterize a CD43 promoter construct that when inserted into the AAVS1 "safe harbor" locus utilizing a zinc finger nuclease specifically drives GFP expression in hematopoietic cells derived from a transgenic ES cell line and faithfully recapitulates endogenous CD43 expression. In addition, using the same gene targeting strategy we demonstrate that constitutive expression of short hairpin RNAs within a microRNA backbone can suppress expression of PU.1, an important regulator of myeloid cell development. We show that PU.1 knockdown cell lines display an inhibition in myeloid cell formation and skewing towards erythroid development. Overall, we have generated a powerful system to track hematopoietic development from pluripotent stem cells and study gene function through hematopoietic specific gene expression and constitutive gene knockdown.

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Introduction

In human ES cells, introduction of stable genetic modifications by homologous recombination has been challenging for many laboratories. Most commonly, transgenes are introduced into ES cells via methods such as viral transduction or plasmid transfection. However, these methods lead to random integration within the genome (Tenzen et al., 2010). Although clones with stable transgene expression can be selected, most clones display transgene silencing upon ES cell

Abbreviations: ES, embryonic stem; GFP, cell, green fluorescent protein; SFD, serum free differentiation; IL-3, media, interleukin-3; EPO, erythropoietin; GM-CSF, granulocyte–macrophage colony stimulating factor; SCF, stem cell factor; TPO, thrombopoietin; IL-6, interleukin-6; RT, reverse transcriptase; PCR, polymerase chain reaction; CA, chicken actin.

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differentiation. For example, even the very well characterized and strong beta globin promoter functions to appropriately induce transgene expression in only a minority of ES cell clones generated using a random integration strategy (Hatzistavrou et al., 2009).

The use of zinc finger nucleases (ZFNs) is one way in which transgenes can be introduced at specific locations within the genome and potentially avoid gene silencing. ZFNs cause a site-specific DNA double strand break and enhance homologous recombination efficiency to introduce a construct of interest using gene targeting (Carroll, 2011). One method to prevent silencing of transgenic constructs is to select genomic loci of constitutively expressed genes for gene targeting. One such site in the human genome is the AAVS1 "safe harbor" locus which encodes the PPP1R12C gene and when targeted with transgenes results in stable gene expression (Dekelver et al., 2010; Hockemeyer et al., 2009). It has been shown that transgenic constructs targeted to the AAVS1 locus maintain expression in hematopoietic cells differentiated from human induced pluripotent stem (iPS) cells when using constitutive promoters (Garçon et al., 2013; Zou et al., 2011) or when using erythroid specific promoters (Chang and Bouhassira, 2012). Therefore, the AAVS1 locus offers a well-characterized site for transgene expression in human pluripotent stem cells.

Here we demonstrate several technical advancements for using the AAVS1 locus to generate both reporter and knockdown constructs in human ES cells. We generate a reporter for hematopoietic cells and a knockdown construct that inhibits myeloid cell formation. One construct is designed to express a green fluorescent protein (GFP) reporter driven by a human CD43 promoter fragment that accurately reflects endogenous CD43 gene expression in hematopoietic cells. The second construct is designed to express short hairpin RNAs driven by the constitutive chicken actin (CA) promoter. By using hairpins that target PU.1, the master regulator of myeloid development, we demonstrate an efficient and stable gene knockdown that leads to a dramatic inhibition of myeloid development. Finally, we show that a simple lipid transfection method can generate a large number of correctly targeted clones much more efficiently and easily than previously published reports using electroporation (Hockemeyer et al., 2009). These techniques provide quick and reliable tools for expressing transgenes and developing reporter and knockdown lines to study hematopoiesis using human pluripotent stem cells.

Materials and methods

Maintenance and hematopoietic differentiation of human ES cells

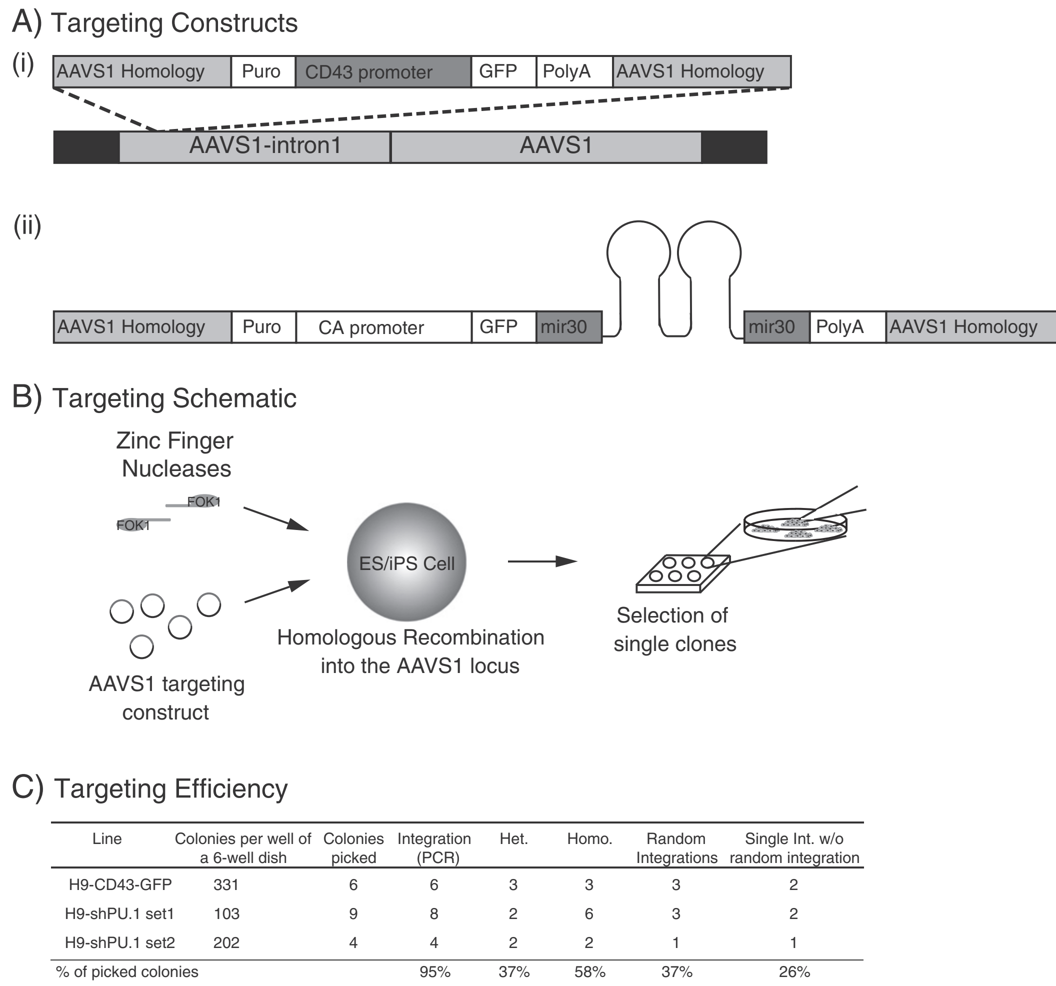
For this study, H9 human ES cells (NIH code WA09, Wicell Research Institute, Madison, WI) were maintained and differentiated as described previously (Mills et al., 2013; Paluru et al., 2013). Briefly, human ES cells were maintained in human ES cell maintenance media in gelatin coated 6-well tissue culture plates (Thermo Fisher Scientific) with irradiated mouse embryonic fibroblast. The composition of the human ES cell media consists of: DMEM/F12 containing 15% knock-out serum replacement, 2 mM glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μM non-essential amino acids, 0.075% sodium bicarbonate, 1 mM sodium pyruvate (all from Invitrogen, Grand Island, NY), 10^{-4} M β-mercaptoethanol (Sigma, St. Louis, MO) and 10 ng/mL human bFGF (Stemgent). The cells were passaged every 3 to 6 days by using TrypLE (Invitrogen). For the monolayer adherent hematopoietic differentiation, the H9 ES cells were feeder depleted by plating onto a 1:3 matrigel (BD Biosciences, Bedford, MA) coated 6-well plate. The differentiation was started 24 to 48 h later when the cells had achieved approximately 70% confluency. The procedure for differentiation has been previously described (Paluru et al., 2013). Hematopoietic progenitors were expanded in cytokine cocktails to drive expansion of myeloid cells: 10 ng/mL IL-3, 100 ng/mL SCF and 200 ng/mL GM-CSF; erythroid cells: 2 units of EPO and 100 ng/mL SCF; and megakaryocytes: 100 ng/mL TPO and 100 ng/mL SCF. All cytokines from R&D Systems (Minneapolis, MN).

Vector construction

ZFNs targeting the AAVS1 locus (Hockemeyer et al., 2009) were synthesized and sub-cloned into a PGK promoter expression vector (ZFN-left and ZFN-right). The CD43 targeting construct was generated using the AAVS1-SA-2A-puro-pA donor plasmid (Addgene, Cambridge, MA). A 2136 base pair sequence from −1386 to +750 of the transcription start site of the human CD43 gene (SPN) was used. This region includes the first exon, first intron and the second exon up to the ATG of the CD43 transcript. This promoter construct was followed by GFP and the rabbit globin poly-adenylation sequence to create AAVS1-SA-2A-puro-CD43 promoter-GFP-PA (Fig. 1A(i)). Generation of the shPU.1 set 1 and set 2 constructs were carried out by cloning pairs of shRNA against PU.1 (Open Biosystems) into a miR-30 backbone (Wang et al., 2007) into the AAVS1-CAGGS-eGFP plasmid (Hockemeyer et al., 2009). The expression of the hairpins is driven by the CA promoter to create AAVS1-SA-2A-puro-CA promoter-GFP-shPU.1-PA (Fig. 1A(ii)). ShPU.1 set1 contains 2 hairpins, one against exon 2 and one against exon 5. Both hairpins in shPU.1 set2 are targeted against two regions of PU.1 in exon 5.

Gene targeting of the AAVS1 locus

Six-well tissue culture plates (Thermo Fisher Scientific) were pre-coated with matrigel (1:3 dilution) (BD Biosciences) followed by plating of 1 × 10^6 irradiated puromycin-resistant mouse embryonic fibroblast cells. H9 ES cells were plated at a density of 1.2–1.8 × 10^6 cells per 6-well dish in human ES cell media containing 10 μM Rho-associated kinase inhibitor Y-27632 (Cayman Chemical, Ann Arbor, MI). The next morning, 3–4 h prior to transfection, the medium was changed to human ES cell medium containing 20 ng/mL bFGF (Stemgent), without penicillin and streptomycin. The targeting vector plasmid (0.6 μg), the ZFN-left plasmid (0.2 μg) and the ZFN-right plasmid (0.2 μg) were added to 100 μL of IMDM (Mediatech) followed by addition of X-tremeGENE9 (3 μL) (Roche Diagnostics). The transfection mixture was gently mixed and incubated at room temperature for 20 min before it was added drop wise into one well of H9 ES cells. The next day, the medium was changed to



Line	Colonies per well of a 6-well dish	Colonies picked	Integration (PCR)	Het.	Homo.	Random Integrations	Single Int. w/o random integration
H9-CD43-GFP	331	6	6	3	3	3	2
H9-shPU.1 set1	103	9	8	2	6	3	2
H9-shPU.1 set2	202	4	4	2	2	1	1
% of picked colonies			95%	37%	58%	37%	26%

Figure 1 AAVS1 safe harbor gene targeting. (A) Schematics of the constructs used in gene targeting. Both constructs contain regions of homology to the AAVS1 locus in the first intron, a puromycin resistance gene (Puro) and a poly-adenylation sequence (PolyA). (i) The CD43-GFP reporter construct contains an approximately 2.1 kb promoter from the human CD43 gene followed by GFP. (ii) The PU.1 knockdown construct contains the chicken actin promoter (CA promoter), GFP, and a pair of short hairpins against PU.1 in the context of the microRNA-30 (mir30) backbone. (B) Schematic of targeting the constructs into the AAVS1 locus. Cells were transfected with plasmids containing the targeting constructs along with the FOK1 zinc finger nucleases. Transfection was done in 6-well plates with lipid transfection. Single clones were then picked and further characterized. (C) Table: Summary of the efficiency of the targeting for the CD43 reporter construct and the PU.1 knockdown constructs. (Het: Heterozygous; Homo: Homozygous; Single Int w/o random integration: Single Integrant without random integration).

human ES cell maintenance medium and 48–72 h post transfection, puromycin (0.5 μg/mL) (Sigma) was added with each media change. Approximately 7 to 10 days later, single clones were manually picked. The clones were passaged and expanded before being screened by Southern blot analysis.

Flow cytometry

Cells were dissociated into single cells by treatment with 0.25% Trypsin/EDTA (Invitrogen) for 3 min followed by addition of fetal bovine serum (Tissue Culture Biologicals). Cells were washed twice with FACS buffer (PBS containing 0.5% BSA and 0.05% sodium azide) and re-suspended in 100 μL of FACS buffer containing the conjugated primary antibody for 15 min at room temperature. A list of primary antibodies can be found in Supplemental Table 1. The cells were washed twice with FACS buffer prior to analysis on a FACSCanto flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometry data were analyzed using the FlowJo (Treestar, San Carlos, CA) software program.

Reverse transcription and quantitative real time polymerase chain reaction (qRTPCR)

Cells were harvested by treatment with 0.25% Trypsin/EDTA (Invitrogen) and cellular RNA was isolated using the RNAeasy micro kit (Qiagen Inc.) as described by the manufacturer's instructions. Random hexamers were used with the Superscript II Reverse Transcriptase (Invitrogen) to synthesize cDNA. PCR reactions were done in triplicate utilizing SYBR-GreenER

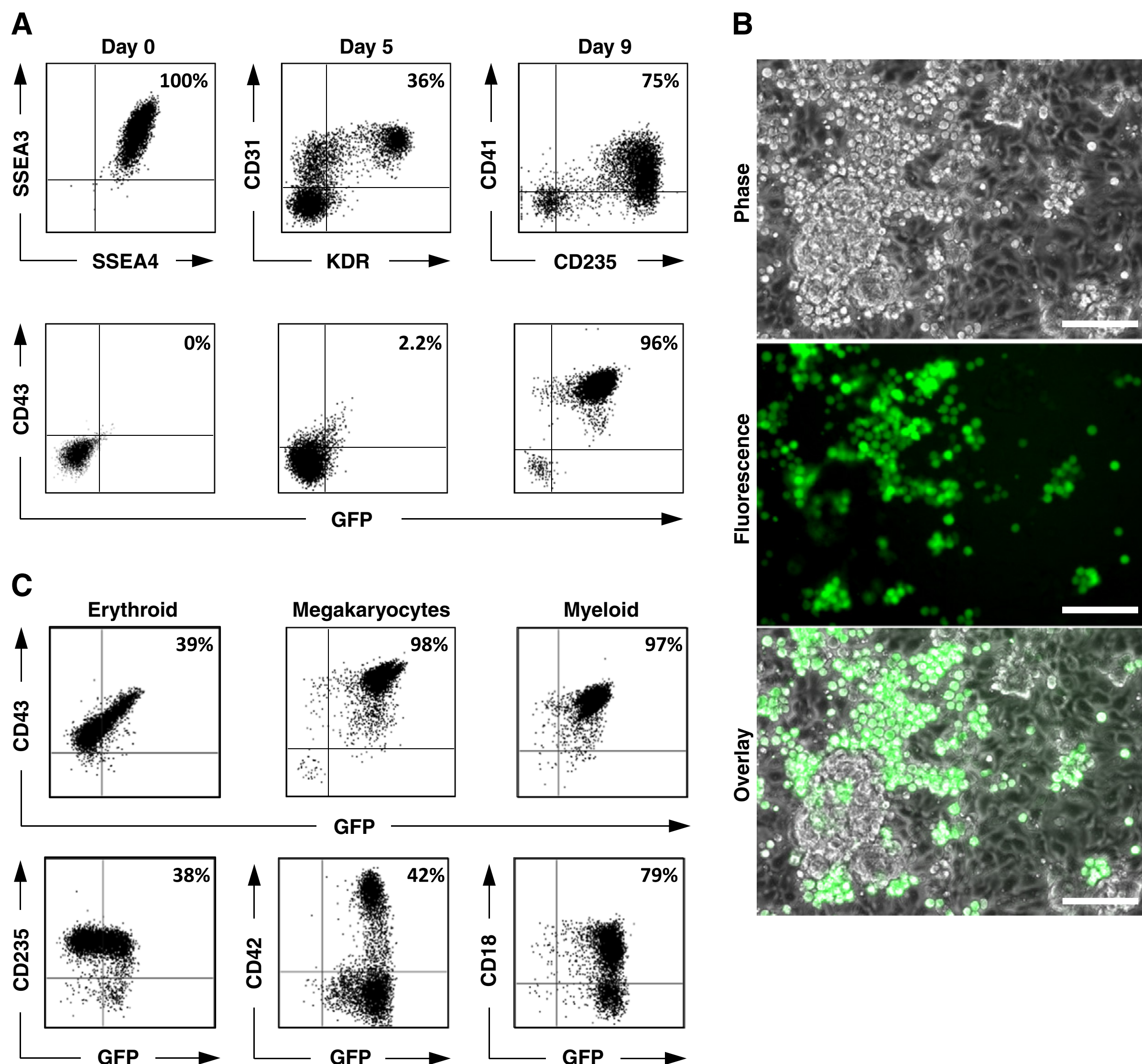


Figure 2 Generation of a CD43-GFP reporter ES cell line. (A) Flow cytometric analysis showing the differentiation of pluripotent CD43-GFP ES cells to hematopoietic progenitors. Cells were analyzed at days 0, 5, and 9 (non-adherent cells only) of differentiation respectively. (Top) Flow cytometric analysis of SSEA3 versus SSEA4, CD31 versus KDR, and CD41 versus CD235. (Bottom) Flow cytometric analysis of CD43 versus GFP expression. (B) CD43-GFP ES cells were differentiated as in A and examined by fluorescence microscopy. Bar is 100 μm. (C) Day 9 non-adherent hematopoietic progenitors from the CD43-GFP ES cell line as described in A were cultured an additional 4 days in cytokine cocktails to promote erythroid, megakaryocytes, and myeloid development. (Top) Flow cytometric analysis of CD43 versus GFP expression. (Bottom) Flow cytometric analysis of CD235 versus GFP, CD42 versus GFP, and CD18 versus GFP expression.

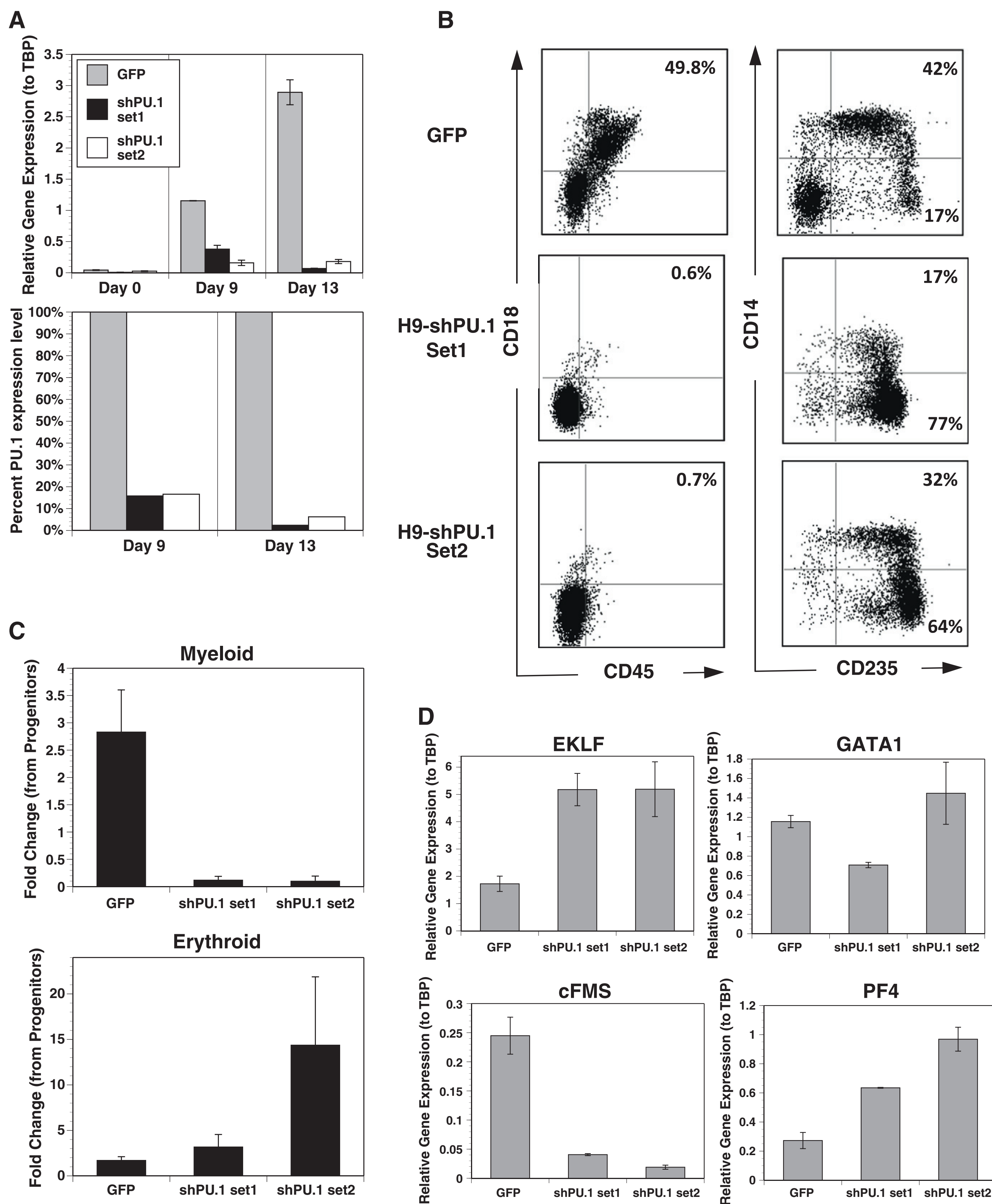
qPCR Master Mix (Roche). The primers that were used in this study can be found in Supplemental Table 2. Serial dilutions of H9 ES cell cDNA were used to generate a standard curve and TBP (Veazey and Golding, 2011) was used as a house keeping gene to determine relative gene expression levels.

Results

To easily quantify hematopoietic development from human ES cells, we created a fluorescent reporter line. The pan-hematopoietic surface marker, CD43 (leukosialin/SPN), is expressed in all hematopoietic lineages beginning at the hematopoietic progenitor stage during in vitro human ES cell differentiation (Vodyanik et al., 2006). In the adult, CD43 is considered to be pan-hematopoietic; however, it is not expressed in mature circulating erythrocytes and a subset of B-cells (Remold-O'Donnell et al., 1987). CD43 was chosen over CD41 or CD45 as these markers are only expressed on subsets of hematopoietic progenitors derived from human ES cells (Vodyanik et al., 2006).

A construct containing the CD43 promoter region driving GFP was targeted to the AAVS1 locus by homologous recombination using the ZFN technology (Fig. 1A). Rather than electroporation, which is known to be toxic to ES cells (Zwaka and Thomson, 2003), a lipid transfection reagent was used. This system is

extremely robust with targeting efficiencies >90% and homozygous targeting in roughly 50% of the clones (Fig. 1C). Approximately 25% of the clones had a single integration into one allele of the AAVS1 locus without additional random integrations as determined by Southern blot (Fig. 1C and Supplemental Fig. 1). Furthermore, a PCR screen demonstrated that these clones are free of ZFN construct integration (Supplemental Fig. 2). These single integrant clones where chosen for further analysis.



To test the specificity of the CD43 reporter construct, the CD43-GFP ES cells were differentiated in vitro into hematopoietic progenitors. The serum free/feeder free in vitro differentiation protocol (Paluru et al., 2013) generates mesoderm after 5 days as indicated by CD31 (PECAM-1) and KDR (VEGF-R2) co-expression (Kennedy et al., 2007), and primitive hematopoietic progenitors appear in the supernatant by day 9 as demonstrated by CD235 and CD41 co-expression (Klimchenko et al., 2009; Vodyanik et al., 2006) (Fig. 2A). Throughout the differentiation, a positive correlation is observed between endogenous CD43 expression, as determined by staining with an anti-CD43 antibody, and GFP expression from the CD43-GFP reporter construct (Fig. 2A and C). In our monolayer differentiation system, the CD43-GFP expressing hematopoietic progenitors are easily visible as they bud off of the monolayer (Fig. 2B).

To demonstrate the stability of transgene expression, the hematopoietic progenitor cells were expanded in liquid culture with the appropriate cytokine cocktails to induce maturation into erythroid (CD235+), megakaryocyte (CD42+), and myeloid (CD18+) lineages (Fig. 2C). In all of the induced hematopoietic lineages, endogenous CD43 expression correlated with GFP expression levels. We observed that the CD235+ erythroid cells eventually expressed lower levels of CD43 and concurrently lost GFP expression. To evaluate the specificity in expression of the CD43 promoter, CD43-GFP ES cells were differentiated into neuroectodermal and definitive endodermal lineages (Supplemental Fig. 3A). Neither of these differentiated cultures expressed GFP (Supplemental Fig. 3B). Together, these data demonstrate that the CD43-GFP transgene is effective as a hematopoietic reporter with GFP expression corresponding to the endogenous expression of CD43.

We next determined whether the AAVS1 targeting system could be used to stably knockdown hematopoietic genes of interest. As a proof of principle, the transcription factor PU.1 was chosen as it is expressed at high levels in monocytes, granulocytes and B lymphoid cells and plays a critical role in the regulation of the myeloid cell fate (Fisher and Scott, 1998). We hypothesized that knockdown of the PU.1 gene would prevent the differentiation of ES cells into myeloid cells. To assure high levels of gene knockdown, we utilized a microRNA based system where two hairpins against a single target are present in the miR-30 backbone (Stegmeier et al., 2005; Sun et al., 2006; Wang et al., 2007). We generated two constructs each containing two short hairpin RNAs (shRNA set1 and set2) against PU.1. The constitutively expressed CA promoter was used to drive GFP followed by the shRNAs (Fig. 1A(ii)). ES cell lines expressing these two constructs (shPU.1 set1 and shPU.1 set2) were generated with efficiencies similar to that achieved with the CD43-GFP construct (Fig. 1C). These data demonstrate the reliability and reproducibility of generating a variety of transgene constructs using this system.

To test the efficiency of gene knockdown, the ES cell lines expressing the shPU.1 constructs were differentiated into hematopoietic cells. The transcription factor PU.1 is not expressed in ES cells at day 0 of differentiation and becomes expressed by day 9 when hematopoietic progenitor cells are generated (Fig. 3A). At this time point, the expression of PU.1 in the two knockdown cell lines was >80% lower than expression in the control GFP-expressing ES cell line. The day 9 hematopoietic progenitor cells were then put in liquid culture for an additional four days in a cytokine cocktail favoring myeloid cell generation. At day 13 of differentiation, the expression of PU.1 in the two knockdown lines was >90% lower than expression in the control cell line (Fig. 3A). These data demonstrate that constitutively expressed AAVS1-targeted knockdown constructs against PU.1 can successfully decrease expression levels throughout differentiation.

To determine the effect of decreased PU.1 levels on hematopoietic cell development, day 9 progenitor cells were put into liquid expansion cultures and analyzed for erythroid (CD235+CD41−) and myeloid (CD18+CD45+) lineages. Both knockdown cell lines expressed almost undetectable levels of CD18 and CD45 compared to ~50% co-expression of these markers on control GFP-expressing cells, indicative of a block in myelopoiesis (Fig. 3B). Furthermore, the absolute yield of myeloid cells in the two knockdown lines was much lower compared to the GFP control cell line (Fig. 3C). These findings were confirmed by gene expression analysis of the myeloid marker cFMS, which was also drastically reduced in the PU.1 knockdown cell lines (Fig. 3D). Analysis of the erythroid lineage demonstrated an ~4-fold expansion in the percentage of CD235+ cells (Fig. 3B) with a concomitant increase in absolute yield of cells, especially in the knockdown line set 2 (Fig. 3C). These findings were confirmed by an increase in gene expression of the erythrocyte transcription factor EKLF in the two knockdown cell lines (Fig. 3D). The pan-hematopoietic marker GATA1 was unaffected and there was a minor increase in the megakaryocyte marker PF4. These data demonstrate that AAVS1-targeted knockdown of PU.1 specifically decreases the myeloid lineage and skews the progenitor cells to an erythroid cell fate.

Discussion

The CD43 promoter fragment we describe here allows tissue specific, controlled expression of a transgene specifically in

Figure 3 Expression of PU.1 short hairpin RNAs inhibit myeloid development. The PU.1 shRNA expressing ES cell lines (H9-shPU.1 set 1 and H9-shPU.1 set 2) along with a H9-GFP expressing control line (GFP) were differentiated into hematopoietic cells and at day 9, non-adherent progenitors were harvested and cultured an additional 4 days in a cytokine cocktail to promote myeloid development. (A) (top) Quantitative RT-PCR of PU.1. expression is reported relative to the housekeeping gene TBP. (Bottom) Percentage of PU.1 expression relative to the control GFP construct in hematopoietic cells at day 9 and day 13 of differentiation. (B) Flow cytometric analysis (CD18 versus CD45 and CD41 versus CD235) on H9-GFP and PU.1 knockdown cell lines at day 13 of differentiation. (C) Absolute cell counts of myeloid (CD18+CD45+) and erythroid (CD41−CD235+) cells in myeloid lineage conditions (SCF 50 ng/mL, GMCSF 200 ng/mL and IL3 10 ng/mL). Cell counts are expressed as fold change from the number of hematopoietic progenitors plated at day 9. (D) Quantitative RT-PCR of hematopoietic lineages genes. Gene expression levels are all reported relative to the housekeeping gene TBP.

cells of the hematopoietic lineages. During the differentiation of the CD43-GFP hematopoietic progenitors into the erythroid lineage it was observed that these cells began to lose the expression of GFP over time in culture. Since endogenous CD43 also decreases, this observation is most likely due to increased sensitivity of the CD43 antibody over GFP and not due to silencing of transgene expression. It has been shown that mature erythrocytes lose expression of CD43, hence this loss of expression is anticipated (Remold-O'Donnell et al., 1987). Importantly, myeloid cells, which maintain CD43 expression in the adult, do not lose GFP expression in the reporter lines while in culture (Fig. 2C). The CD43 reporter represents a valuable tool to easily follow hematopoietic specification from ESCs in live cultures with specificity that rivals CD41 or CD45 as all hematopoietic progenitors are CD43+ (Vodyanik et al., 2006).

In mouse models, a homozygous loss of PU.1 is embryonic lethal (Scott et al., 1994) or if mice are born alive, they die shortly after birth (McKercher et al., 1996). Mice that are born are found to lack any mature B and T cells, macrophages and neutrophils but have erythrocytes and megakaryocytes (McKercher et al., 1996) indicating that PU.1 is required for myeloid cell lineage development. A heterozygous loss of PU.1 is non-lethal, however it leads to a loss in the formation of multipotent myeloid progenitors (Scott et al., 1994). We demonstrate that a >80% knockdown of PU.1 in human hematopoietic cells results in a loss of myeloid lineage cells, phenocopying what occurs with a loss of PU.1 in the mouse. The differentiation of ES cells into primitive hematopoietic cells can be considered to mimic human development, thus our data suggests that during early human hematopoiesis PU.1 is also required for myeloid lineage development.

The advent of new technologies such as TALENs and CRISPRs offer extremely powerful methods for enhanced gene editing (Gaj et al., 2013). Indeed, these technologies have been shown to target the AAVS1 locus with similar efficiencies as ZFNs (Hockemeyer et al., 2011; Mali et al., 2013). However, targeted gene editing and transgene expression to a novel site will still require targeting vector construction, the development of screening protocols and the labor intensive screening of potentially many ES cell clones which will vary depending on the targeting construct and gene of interest. The advantage of the system we describe here is that the same targeting vector and screening methodology can be used for any construct, allowing the quick and easy generation of transgenic pluripotent stem cell lines with high efficiency. In addition, we have adapted the use of a simple lipid transfection reagent to introduce our targeting constructs which allows the generation of sufficient targeted clones from a single well of a six well dish.

We have shown that it is possible to have site-specific integration and stable expression of a transgene throughout hematopoietic differentiation. This system represents a powerful and flexible tool to study hematopoiesis, and for the case of gene knockdown to study the requirement of a particular gene in the context of any cell type where there is a differentiation protocol available. Both the constructs described here have the potential for use in future studies involving transgene expression in differentiated progeny from pluripotent stem cells. Together, these techniques allow the easy manipulation of gene expression and will strengthen the use of ES cells as a model system for the study of early human development and disease.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.02.004.

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