Efficient CRISPR-Cas9 mediated gene disruption in primary erythroid progenitor cells

The study of isolated primary progenitor cells offers great insight into developmental biology and human disease. In particular, *ex vivo* culture of isolated primary erythroid progenitor cells replicates the differentiation events that occur during *in vivo* erythropoiesis. Herein we report a high-efficiency method for CRISPR-Cas9 mediated gene disruption in isolated primary erythroid progenitor cells. We use this method to generate the novel result that *Lmna* is required in terminal erythroid differentiation.

Terminal erythroid differentiation is initiated by the binding of Erythropoietin (Epo) to Epo receptors on the surface of CFU-E erythroid progenitors, leading to 3 to 6 terminal divisions the induction of ~600 genes and repression of thousands of others in a defined temporal

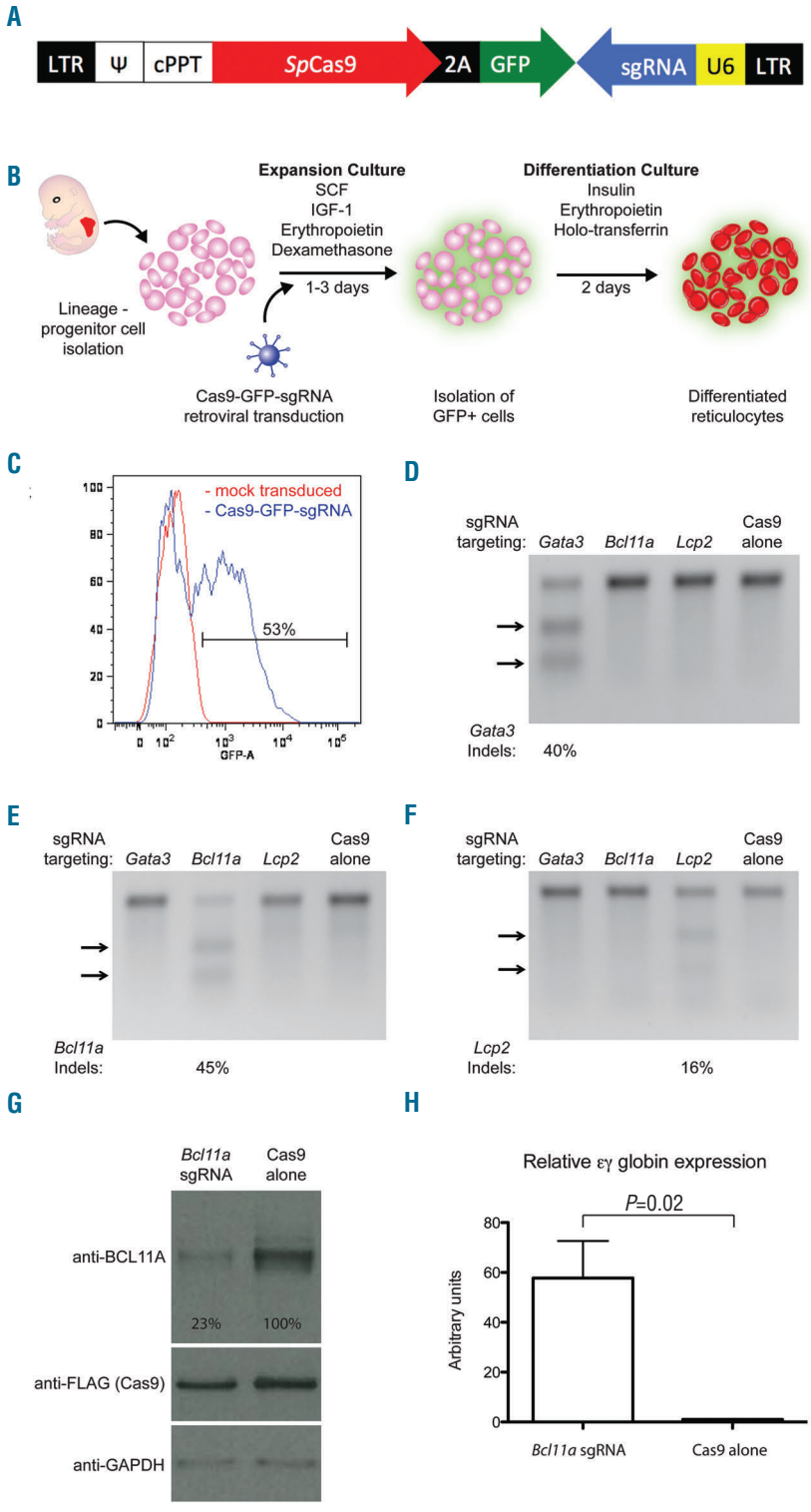

Figure 1. CRISPR-Cas9 expression in primary fetal liver erythroid progenitors mediates disruption of multiple genes and loss of *Bcl11a* gene function. (A). Diagram of MSCV-based retroviral vector co-delivering Cas9-GFP and sgRNA. (B) Schematic of experimental procedure including cytokines used in different culture phases. (C) Flow cytometry assessment of efficiency of Cas9 and sgRNA delivery to fetal liver erythroid progenitor cells as quantified by GFP expression. (D-F) Surveyor nuclease assay of gene disruption efficiency in fetal liver erythroid progenitor cells in the *Gata3*, *Bcl11a*, and *Lcp2* genes following delivery of Cas9 with indicated sgRNAs or of Cas9 alone. Arrows denote cleavage products and lanes without indel quantification indicate less than 1% disruption efficiency. (G) Western blot assessing level of BCL11A protein and FLAG-tagged Cas9 in fetal liver erythroid progenitor cells following delivery of Cas9 with and without sgRNA targeting *Bcl11a*. Relative expression differences of BCL11A protein quantified by densitometry. (H) RT-PCR quantification of relative εγ globin mRNA levels in reticulocytes following delivery of Cas9 with *Bcl11a* sgRNA or Cas9 alone. Data are represented as the mean of 3 biological replicates and error bars denote standard deviation. *P*-value calculated using Student's *t*-test.

order. Concomitantly there are major changes in cytoskeletal and nuclear architecture, including condensation of the nucleus to about one-tenth of its original volume and, only in mammals, enucleation.[1,2,3] Numerous human diseases cause disordered erythropoiesis and result in significant morbidity and mortality. In many cases the underlying mechanisms of the disease are incompletely understood, and more importantly, the

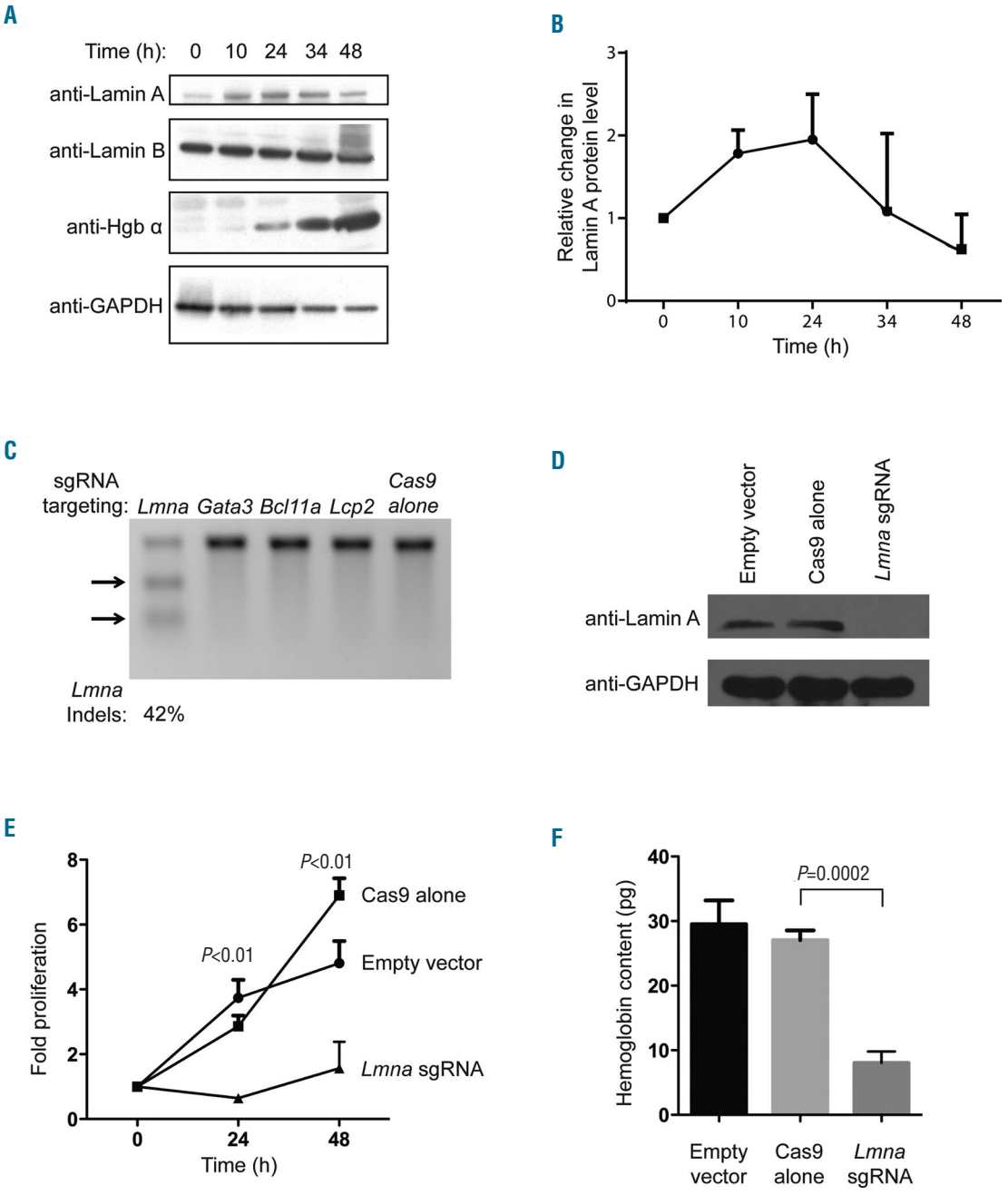

Figure 2. Dynamic regulation of Lamin A during erythroid differentiation and impaired terminal erythroid differentiation following CRISPR-Cas9-mediated *Lmna* disruption. (A) Western blot of Lamin A protein levels following transition of fetal liver erythroid progenitors to differentiation culture. One million cells were loaded per lane. (B) Densitometry quantification of relative changes in Lamin A protein levels following transition of fetal liver erythroid progenitors to differentiation culture. Each data point represents the mean of 3 biological replicates with error bars denoting standard deviation. (C) Surveyor nuclease assay of disruption efficiency of the *Lmna* gene in fetal liver erythroid progenitor cells following delivery of Cas9 with indicated sgRNAs or Cas9 alone. Arrows denote cleavage products and lanes without indel quantification indicate less than 1% disruption efficiency. (D) Western blot assessing levels of Lamin A protein in fetal liver erythroid progenitor cells following delivery of empty vector, or Cas9 with and without a sgRNA targeting *Lmna*. (E) Proliferation of fetal liver erythroid progenitors in differentiation medium following delivery of empty vector, or Cas9 with and without *Lmna* sgRNA. Data points represent the mean of 3 biological replicates and error bars denote standard deviation. *P*-values comparing Cas9 with *Lmna* sgRNA to other conditions were calculated using Student's *t*-test. (F) Hemoglobin content per reticulocyte generated from fetal liver erythroid progenitors following delivery of empty vector, or Cas9 with and without sgRNA targeting Lmna. Data represent the mean of 3 biological replicates and error bars denote standard deviation. *P*-value calculated using Student's *t*-test.

identification of the disease modifier genes and their function in terminal erythropoiesis is incomplete.[4]

Ex vivo culture and differentiation of isolated primary mouse erythroid progenitor cells faithfully mimics the cellular events that occur during *in vivo* erythropoiesis, including proliferation, upregulation of erythroid-specific genes and cell surface markers, accumulation of hemoglobin, and enucleation.[5] RNA interference has been used for the study of gene function in erythropoiesis, but the use of small hairpin RNAs is complicated by the difficulty in predicting the efficiency of gene knockdown and non-specific effects.[6] Several genetic determinants of erythropoiesis have been assessed using erythroid progenitors isolated from gene knock-out mice,[7,8] but this is a laborious process involving *de novo* generation of individual mouse lines that is not amenable to the high-throughput assessment of candidate genes.

CRISPR-Cas9 technology has allowed rapid genetic loss of function in many organisms *via* nuclease-mediated gene disruption.[9,10] Intracellular delivery of the prokaryotic Cas9 nuclease complexed with a single-guide RNA (sgRNA) molecule results in sgRNA-directed cleavage of a specific genomic site by Cas9. The DNA double-strand break induced by Cas9 cleavage is then frequently repaired by the endogenous non-homologous end joining (NHEJ) DNA repair pathway, resulting in insertion and deletion (indel) mutations that disrupt gene expression.[9,10]

However, the successful use of CRISPR-Cas9 in isolated primary erythroid cells has not yet been described. Herein we report on a highly efficient method for delivering the *Streptococcus pyogenes* Cas9 protein and a sgRNA to isolated primary erythroid progenitor cells. Cas9 and sgRNA delivery with this method results in robust genetic loss of function through gene disruption, and we use this method to identify a novel gene required in terminal erythropoiesis.

To deliver Cas9 and a sgRNA to primary erythroid progenitor cells, we constructed a murine stem cell virus (MSCV)-based retroviral vector co-expressing Cas9 and green fluorescent protein (GFP) linked by a self-cleaving 2A peptide to mediate equimolar expression of Cas9 and GFP from a single Pol II promoter cassette. This vector also expresses a sgRNA from a Pol III promoter cassette (Figure 1A). Using this vector we transduced primary lineage negative erythroid progenitor cells purified from E14.5 mouse fetal livers, and cultured them in an erythroid progenitor expansion media containing cytokines that promote proliferation but not differentiation. GFP positive cells were isolated by fluorescence activated cell sorting (FACS) between 24 to 72 hours post-transduction depending on the desired degree of cell expansion (Figure 1B). Importantly, our retroviral vector delivering Cas9 and sgRNA was capable of high efficiency transduction, such that the majority of the progenitor cell population was successfully transduced (Figure 1C). GFP positive cells were then placed in erythroid differentiation medium for 48 hours (Figure 1B).

To assay gene disruption efficiency we co-delivered Cas9 with three different sgRNAs (*Online Supplementary Table S1*), one targeting the start codon of the *Gata3* gene, which encodes a T-lymphocyte specific transcription factor, one targeting the 5' end of the *Lcp2* gene, which encodes a signal transduction adaptor protein, and one targeting the start codon of the *Bcl11a* gene, which encodes a multilineage transcription factor that is expressed in erythroid precursor cells. For all 3 vectors, transduction efficiency was greater than 50% when performing FACS for GFP positive cells (*data not shown*). Using the Surveyor nuclease assay on GFP positive cells, we detected robust generation of indels in each of these genes (Figures 1D-F).

As the Surveyor nuclease assay suggested the presence of a mixed population of bi-allelically disrupted, haploinsufficient, and wild-type cells, we sought to assess if the observed gene disruption rates resulted in the loss of protein expression and an observable phenotype. To determine if gene disruption through indel generation resulted in the loss of protein expression, we assessed levels of the protein encoded by the *Bcl11a* gene and found nearly 80% decreased expression in cells transduced with vector expressing Cas9 and a sgRNA targeting *Bcl11a* (Figure 1G), indicating the percent indels reflected by the Surveyor assay only provided a minimum estimate of gene inactivation. As *Bcl11a* controls the developmental switch to adult hemoglobin in mammalian erythropoiesis and mouse models genetically deficient in *Bcl11a* have elevations in εγ globin gene expression,[7] we determined whether primary erythroid progenitors had elevations in εγ globin gene expression following disruption of the *Bcl11a* gene. In reticulocytes differentiated from progenitor cells transduced with vector expressing Cas9 and a sgRNA targeting *Bcl11a*, there was a greater than fifty-fold increase in εγ globin mRNA, replicating the phenotype observed in mice genetically deficient in *Bcl11a* (Figure 1H).

We next used this system to identify a novel regulator of terminal erythroid differentiation. As prior studies of erythropoiesis had not placed significant emphasis on structural proteins, we analyzed expression data for all genes encoding such types of proteins. *Lmna*, the gene encoding the alternatively spliced nuclear envelope proteins Lamins A and C, exhibited the greatest increase in expression during terminal erythroid differentiation, with a 4 to 6-fold increase in relative expression quantified by RNA-seq methods.[2] This was in contrast to Lamin B, a protein that complexes with Lamins A and C to support nuclear membrane architecture and control transcriptional regulation, but is downregulated during terminal erythroid differentiation.[3,11,12] Interestingly, the Lamin A protein exhibited a marked initial increase during terminal differentiation followed by a marked decrease (Figure 2A,B), whereas Lamin C protein levels were not as dynamic (*Online Supplementary Figure S2*).

We designed a sgRNA targeting the start codon of *Lmna* (*Online Supplementary Figure S1*). Retroviral vector transduction resulted in the successful delivery of Cas9 and the *Lmna* sgRNA to greater than 50% of isolated primary erythroid progenitors, as assessed by the frequency of GFP positive cells when performing FACS (*data not shown*). The delivery of Cas9 and the sgRNA targeting *Lmna* to primary erythroid progenitors resulted in the efficient introduction of indels in the *Lmna* gene (Figure 2C), and in the reduction of Lamins A (Figure 2D) and C (*Online Supplementary Figure S2*) protein levels. Progenitors with a disrupted *Lmna* gene showed impaired proliferation (Figure 2E) as well as a decreased ability to accumulate hemoglobin (Figure 2F). Many cells underwent apoptosis (*data not shown*). There was no difference in upregulation of cell surface markers CD71 and Ter119 (*Online Supplementary Figure S3*), which are relatively early events during terminal erythroid differentiation.[1] These results are the first demonstration that expression of *Lmna* is dynamically regulated during erythropoiesis and that it plays a significant role in terminal red cell dif-

ferentiation. Although an anemia phenotype has not been reported for *Lmna*$^{-/-}$ mice, they do have decreased growth by 3 weeks and die by 8 weeks of life;[13] it will be interesting to determine whether young *Lmna*$^{-/-}$ mice do exhibit an anemia phenotype under both normal and stress erythropoiesis conditions.[14] Further biochemical analyses will be instrumental in determining the underlying mechanism by which *Lmna* regulates terminal erythroid differentiation.

The method we developed for using CRISPR-Cas9 to study genetic loss of function in isolated primary erythroid progenitor cells provides a broadly generalizable, rapid, and facile assessment of gene function in red blood cell development, with a total duration of 3 to 5 days from progenitor cell isolation to phenotypic assessment. Although off-target effects of the CRISPR-Cas9 system are of concern, high-throughput sequencing or array CGH methods can be used to identify off-target cleavage events, and rescue experiments using cDNAs of targeted genes where codons have been substituted to ablate sgRNA binding without changing the amino acid sequence can confirm whether the observed phenotype is due to on-target or off-target gene disruption. Additionally, the use of novel Cas9 variants with high specificity will mitigate many of these concerns.[15] Our system has significant strengths in the high transduction rate of our viral vector co-expressing Cas9 and a sgRNA, and the high efficiency with which gene disruption can be achieved, both of which are key considerations when studying rare and difficult-to-isolate cell populations such as hematopoietic progenitors. Indeed, the ability to achieve high-efficiency gene disruption using this system allows the possibility of robust large-scale genetic loss-of-function screening in erythropoiesis.

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