Flicking the switch: adult hemoglobin expression in erythroid cells derived from cord blood and human induced pluripotent stem cells

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So far, only modest degrees of adult hemoglobin expression in red blood cells (RBCs) derived *in vitro* from cord blood cells, human embryonic stem cells (hESCs) or induced pluripotent stem (hiPS) cells have been reported. In this issue of Haematologica, Trakarnasanga *et al.*[1] demonstrate that forced expression of KLF1 and BCL11A induces switching to adult hemoglobin expression in erythroid cells derived from cord blood and hiPS cells.

The demand for a safe and unlimited source of adult RBCs

Red blood cell transfusions are, in many cases, life-saving treatments for patients with anemia, either due to underlying genetic disease, as is the case for thalassemia patients, or in the event of sudden blood loss. Globally, around 85 million units of RBCs, derived from 0.5-0.7 liters of donor blood per unit, are transfused each year.[2] Hence, the demand for donor blood is high, but availability is limited and this may cause shortages for patients, in particular for those with rare blood groups. Indeed, complications such as the potential for transmission of (unknown) infectious diseases and adverse immune reactions further emphasize the need to develop safe and reliable alternatives to donor blood. Ideally, controlled *in vitro* production of fully functional mature adult RBCs should lead to unlimited supplies of safe and universal transfusion units. The availability of human RBCs derived *in vitro* from hematopoietic stem/progenitor cells (HSPCs) in clinically useful quantities would have a major impact on transfusion medicine worldwide. For the moment, there are no such *in vitro*-produced alternatives to donor-derived human RBCs. Although in recent years the development of systems for production of RBCs *in vitro* has progressed enormously, many challenges remain in translating this cellular therapy to the clinic.

In vitro generation of mature adult red blood cells

For many years, there have been intense efforts aimed at establishing *in vitro* differentiation of human erythroid cells starting from HSPCs originating from peripheral blood, cord blood, hESCs or hiPS cells. More recently, increasingly efficient conditions have been developed to induce erythroblast expansion, differentiation, enucleation and maturation, mimicking many aspects of *in vivo* erythropoiesis (Figure 1). The first heroic efforts by Migliaccio *et al.*[3] and Fibach *et al.*[4] to grow and differentiate erythroid progenitors *in vitro* date back to the late 1980s. They used cells derived from human bone marrow and adult peripheral blood. Subsequently, several groups have adopted these protocols and modified the procedures, with the aim of obtaining enucleated and fully functional adult RBCs (reviewed by Mazurier *et al.*[5] and Migliaccio *et al.*[6]). Giarratana *et al.* have demonstrated that red cells can be generated efficiently starting from HSPCs derived from human peripheral blood.[7] The cells produced in this study were mature reticulocytes, they expressed adult hemoglobin, they bound oxygen in a reversible manner, and their rheological properties were similar to those of reticulocytes released *in vivo* into the circulation of healthy individuals. Giarratana and colleagues also showed for the first time that cultured RBCs persisted for several weeks after transfusion into a human volunteer.[7] Although very promising, HSPCs derived from peripheral blood have a limited proliferative capacity and they exhaust quickly, precluding their application to large-scale production of RBCs.

Red blood cells generated from other sources still need to be fully characterized. Umbilical cord blood is a rich source of highly proliferative HSPCs. It has been demonstrated that an HSPC from cord blood can produce up to ten times more RBCs than one derived from adult peripheral blood.[8] Cord blood RBCs derived *in vitro* share many characteristics with fetal RBCs, which will likely limit their utility for transfusion of adults. In addition, current technology does not enable unlimited expansion of cord blood HSPCs. Since cord blood can only be collected once from every newborn, it is not a sustainable cell source for large-scale production of RBCs. In contrast, hESCs and hiPS cells can be maintained indefinitely in culture. Lu and colleagues found that erythroid cells derived from hESCs can termi-

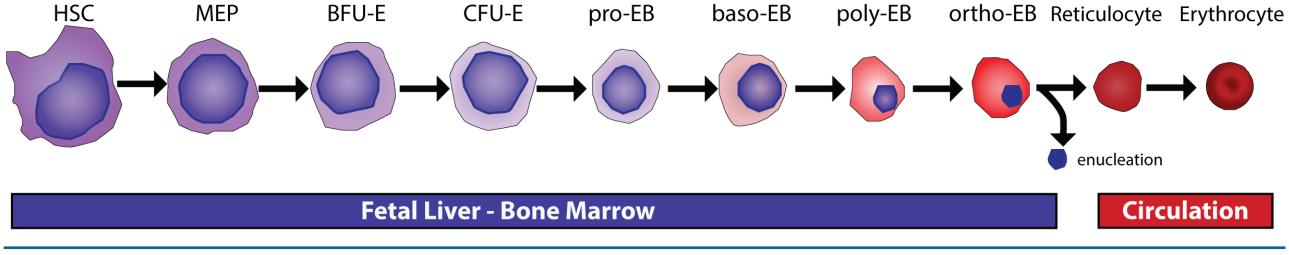

Figure 1. Erythropoiesis. Schematic representation of erythroid development from the hematopoietic stem cell (HSC) via several committed progenitor stages to mature erythrocyte. MEP: megakaryocyte-erythroid progenitor; BFU-e: burst forming unit erythroid; CFU-e: colony forming unit erythroid; EB: erythroblast; baso: basophilic; poly: polychromatic; ortho: orthochromatic.

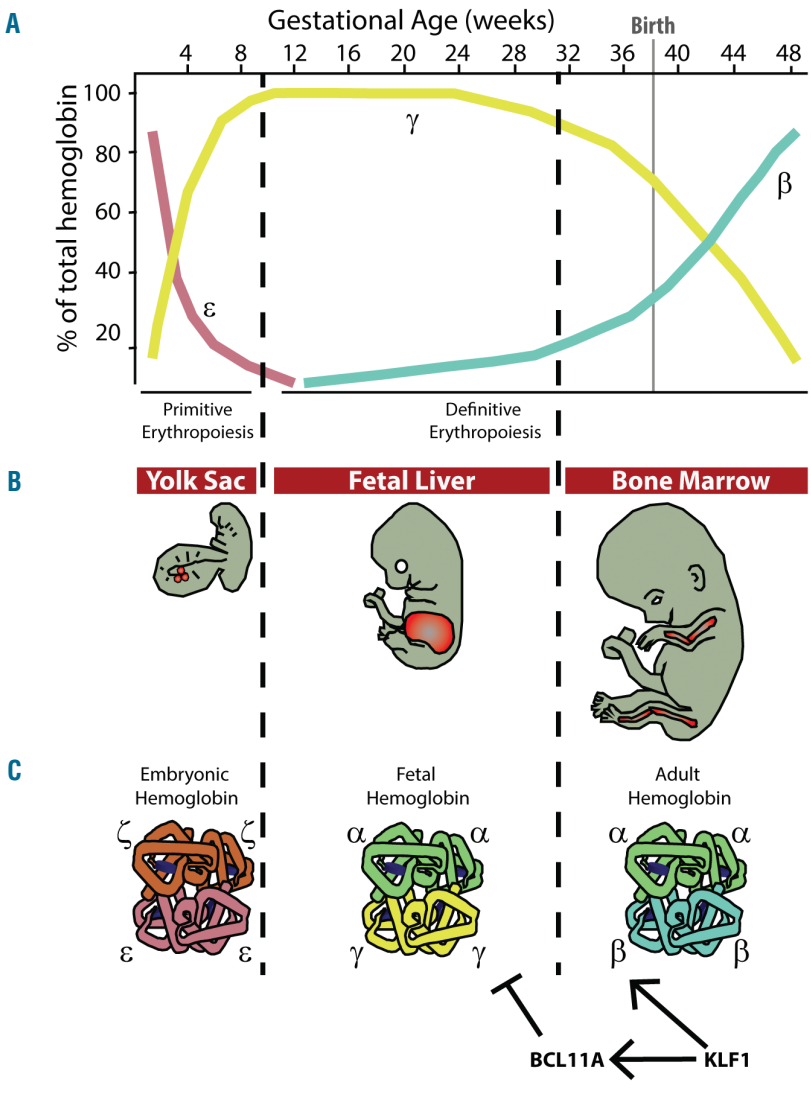

Figure 2. Human erythropoiesis and developmental pattern of hemoglobin expression. (**A**) Hemoglobin switching in man. Around week 6 of gestation, embryonic globin (ε) is silenced and fetal globin (γ) starts to be expressed. Perinatally the switch to adult globin (β) occurs. For the α-like globins, a single switch from the embryonic (ζ) to adult (α) globin occurs (not shown). (**B**) Major anatomical sites of hematopoiesis during development. Erythropoiesis occurs in the blood islands of the yolk sac in the first 8 weeks of gestation, then in the fetal liver between 8 and 32 weeks, and finally in the bone marrow from 32 weeks on. Around birth the spleen serves as a transient erythropoietic organ (not shown). (**C**) Structure of the main human hemoglobins expressed during development. Embryonic globin ($\zeta_2\varepsilon_2$; HbE Gower-1); fetal hemoglobin ($\alpha_2\gamma_2$; HbF) and adult hemoglobin ($\alpha_2\beta_2$, HbA). KLF1 and BCL11A are two transcription factors with key roles in the developmental regulation of hemoglobin expression. In particular, KLF1 activates β-globin and BCL11A, which in turn represses γ-globin expression.

nally differentiate *in vitro*,[9] and hiPS cells have also been shown to possess hematopoietic potential, using methods similar to those for generating erythroid cells from hESCs.[10]

Between the two sources, hiPS cells present two major advantages: they pose no ethical barriers regarding the origin of the cells, and they can be selected for phenotypes of interest. Even though in recent years we have seen great advances in the production of RBCs starting from hESCs and hiPS cells, the procedure needs to be further optimized before clinical applications can even be considered. The generation of adult RBCs from these cell sources presents considerable challenges. RBCs generated from these cells predominantly express embryonic hemoglobins and only modest degrees of enucleation have been reported.[11,12] To achieve development of a clinical transfusion product, this system will have to be modified to enable generation of RBCs approximating as closely as possible normal adult RBCs. In addition, rigorous characterization will be required to establish safety, efficacy and lack of immunogenicity.

Switching to adult hemoglobin expression

As highlighted above, one of the outstanding issues is that RBCs derived *in vitro* from cord blood or hIPS cells produce mainly embryonic and fetal hemoglobins, composed of ζ-globin or α-globin teamed up with ε- globin or γ-globin.[13] In contrast, over 97% of hemoglobin in adult RBCs is adult hemoglobin, composed of α-globin and β-globin (Figure 2). Trakarnasanga *et al.* addressed this issue by transducing embryonic/fetal hemoglobin-expressing cells with KLF1 and BCL11A, two key developmental regulators of hemoglobin expression *in vivo*.[14,15] They found that the presence of both transcription factors above a threshold level is sufficient to induce expression of β-globin to levels equivalent to those observed in adult erythroid cells. This result supports the model of KLF1-BCL11A co-operation in switching to production of adult hemoglobin (Figure 2)[14] and might, therefore, be taken for granted by the scientific community. It is, nevertheless, important to demonstrate experimentally that the combined activity of KLF1 and BCL11A results in production of adult hemoglobin in cells that are intrinsically programmed to express embryonic/fetal hemoglobin. Furthermore, while in adult erythroid progenitor cells KLF1 drives expression of BCL11A (Figure 2),[14] this does not appear to be the case in cells used by Trakarnasanga *et al.*, suggesting that KLF1 co-

operates with an as yet unknown co-factor to fully activate BCL11A expression in adult erythroid progenitors. At this stage, we can only speculate about the identity of this co-factor. Given its role in adult erythropoiesis and repression of embryonic/fetal hemoglobin expression, the SOX6 transcription factor is an attractive candidate.[16,17] Alternatively, post-translational modifications and subcellular localization of KLF1 could be involved.[18] Thus, the current work sheds some light on the conundrum that KLF1 is already a functional transcriptional activator in embryonic erythroid cells which do not express adult hemoglobin.[19] Furthermore, it provides leads for follow-up experiments, such as the introduction of factors in cultured human fetal liver erythroid progenitor cells as a tool to study the molecular mechanism of hemoglobin switching.

Further steps towards in vitro *production of transfusion-grade mature adult red blood cells*

In the study by Trakarnasanga *et al.*, the cells were virally transduced to increase KLF1 and BCL11A levels. Integration of such transgenes into the host genome raises safety concerns related to the potential impact on expression of endogenous genes. The production of RBCs should be oriented towards techniques which either avoid transgene integration altogether, or control the sites of integration with precision to minimize the risk of adverse effects, including neoplastic transformation. Recently developed genome editing technologies such as TALEN and the CRISPR/CAS9 system,[20] combined with whole genome sequencing of the cells selected for RBC production, could be applied to overcome these safety concerns.

Collectively, recent progress suggests that transfusion of patients with *in vitro*-produced RBCs may become a reality in the future. Nevertheless, there are still several hurdles to be overcome before this goal can be achieved. The identification of an unlimited source of clinical grade HSPCs is one such obstacle. Another is to produce the large quantities of RBCs needed for every single transfusion unit. Current efforts have focused on generation of RBCs on a laboratory scale, but in order to meet the demand for clinical applications, much more efficient processes will have to be developed. The design of bioreactors optimized for this purpose might ultimately create conditions that induce erythropoiesis and at the same time promote maintenance of HSPCs, allowing sustained production of fully functional adult RBCs at reasonable costs. The work described by Trakarnsanga *et al.* represents a small, but significant, step towards this goal.

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