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Ankyrin and Band 3 Differentially Affect Expression of Membrane Glycoproteins But Are Not required for Erythroblast Enucleation

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Abstract

During late stages of mammalian erythropoiesis the nucleus undergoes chromatin condensation, migration to the plasma membrane, and extrusion from the cytoplasm surrounded by a segment of plasma membrane. Since nuclear condensation occurs in all vertebrates, mammalian erythroid membrane and cytoskeleton proteins were implicated as playing important roles in mediating the movement and extrusion of the nucleus. Here we use erythroid ankyrin deficient and band 3 knockout mouse models to show that band 3, but not ankyrin, plays an important role in regulating the level of erythroid cell membrane proteins, as evidenced by decreased cell surface expression of glycophorin A in band 3 knockout mice. However, neither band 3 nor ankyrin are required for enucleation. These results demonstrate that mammalian erythroblast enucleation does not depend on the membrane integrity generated by the ankyrin-band 3 complex.

Keywords

Erythropoiesis; Enucleation; Ankyrin; Band 3; Glycoprotein

1. Introduction

Red blood cells are continuously replenished; in humans their half-life is 120 days. Erythropoietin (Epo), a cytokine produced by the kidney in response to low oxygen pressure, is the principal regulator of red blood cell production. Epo binds to cognate receptors on Colony Forming Unit Erythroid (CFU-E) progenitor cells and activates the JAK2 protein tyrosine kinase and several downstream signal transduction cascades including the Stat5, PI3K/Akt, and Ras-MAP kinase pathways [1]. These prevent CFU-E apoptosis and stimulate its terminal proliferation and differentiation; during the ensuing three days each CFU-E produces 30–50 erythroblasts that undergo terminal cell cycle exit,

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chromatin and nuclear condensation, and enucleation forming reticulocytes that are released into the circulation.

Multiple proteins and signaling pathways are involved in the enucleation process, including histone deacetylation, actin polymerization, cytokinesis, cell-matrix interactions, specific microRNAs, and vesicle trafficking [2,3]. Specifically, histone deacetylase 2 (HDAC2) is required for chromatin condensation and subsequent enucleation of mouse fetal liver erythroblasts[4]. Enucleation also requires Rac GTPases and their target formin mDia2 to form the contractile actin ring between the incipient reticulocyte and extruding nucleus [5].

Evidences from early studies indicated that enucleation is a process of cytokinesis where various proteins in membrane and cytoskeleton network are involved [6,7,8,9]. The organization and functions of this network have been well established. In brief, the heterodimeric α and β-spectrins self-associate into $\alpha_2 \beta_2$ tetramers, which are connected by actins to form pentagonal or hexagonal lattices that underlie the plasma membrane [10]. The ankyrin-band 3 complex connects the spectrin-based skeleton to the plasma membrane and helps maintain the vertical and horizontal integrities of the mature erythrocyte. Mice with homozygous mutations of ankyrin (*nb/nb* mice) exhibit nearly complete ankyrin deficiency and severe hemolytic anemia [11]. The same phenotype is also observed in mice with a targeted depletion of band 3 [12]. On the other hand, mice carrying spontaneous mutations of α-spectrin (*sph/sph* mice) or β-spectrin (*ja/ja* mice) survive to birth but quickly die of severe hemolytic anemia [13]. In humans, functional disruption of these proteins frequently leads to hereditary spherocytosis (HS), hereditary elliptocytosis (HE) or hereditary pyropoikilocytosis (HPP) [14]. In light of the significance of the ankyrin-band 3 complex in the integrity of the red cell membrane, together with the fact that nuclear condensation occurs throughout the vertebrate phylum but that enucleation is only observed in mammals, we hypothesized that a deficiency of ankyrin or band 3 could affect the extent and/or rate of mammalian erythroblast enucleation.

Here we use our well-established *in vitro* mouse fetal liver erythroblasts culture system [5] to investigate enucleation in *nb/nb* and band 3 targeted knockout mice. We show that ankyrin and band 3 play distinct roles in the expression of erythroid cell surface protein glycophorin A, but that neither is required for the enucleation process. These results provide definitive evidence that the membrane-cytoskeleton integrity formed by the ankyrin-band 3 complex is not required for mammalian erythroblast enucleation.

2. Materials and Methods

2.1. Purification and culture of mouse fetal liver erythroblasts

Purification and culture of E13.5 TER119 negative mouse fetal liver erythroblasts were described previously [4,5,15]. Briefly, fetal liver cells were isolated from E13.5 embryos of different genotype and mechanically dissociated by pipetting in PBS containing 10% FBS (GIBCO). Single-cell suspensions were prepared by passing the dissociated cells through 70μm and 25μm cell strainers. Total fetal liver cells were labeled with biotin-conjugated anti-TER119 antibody (1:100) (BD Pharmingen), and TER119 negative cells were purified through a StemSep column according to the manufacturer's instructions (Stem Cell Technologies). Purified cells were seeded in fibronectin-coated wells (BD Pharmingen) at a cell density of 1×10^5/ml and cultured in IMDM containing 15% FBS (StemCell Technologies), 1% detoxified bovine serum albumin (BSA), 200 μg/ml holo-transferrin (Sigma), 10 μg/ml recombinant human insulin (Sigma), 2 mM L-glutamine, 10^{-4} M β-mercaptoethanol, and 2 U/mL Epo (Amgen).

2.2. Flow cytometry analysis

Flow cytometric analysis of *in vivo* fetal liver erythroid cells and analysis of the differentiation status of cultured mouse fetal erythroblasts was described previously[5]. Briefly, fetal liver cells were immunostained with phycoerythrin (PE) conjugated anti-TER119 (1:200) (BD Pharmingen) and fluorescein isothiocyanate (FITC) conjugated anti-CD71 (1:200) (BD Pharmingen) antibodies, for 15 min at room temperature. Propidium iodide was added to exclude dead cells from analysis. For enucleation analysis, cells were stained with 10 μg/ml Hoechst 33342 (Sigma), in addition to fluorophore-conjugated antibodies, for 15 min at room temperature.

2.3. Statistical analysis

Statistical analysis was performed using GraphPad Prism. The student's t-test was used to determine the significance of experimental groups versus control ones.

3. Results

To directly investigate the roles of ankyrin in mammalian erythroblast enucleation, we first evaluated the extent of enucleation *in vivo* in *nb/nb* mice during fetal liver erythropoiesis, since more than 90% of the fetal liver cells are erythroid [5]. Specifically, total fetal liver cells from embryonic day 14.5 (E14.5) mice were purified and stained with erythroid-specific TER119 (antigen associated with erythroid cell-surface glycophorin A) and the cell permeable DNA staining dye Hoechst 33342 for flow cytometry analysis. Figure 1A shows that the expression levels of TER119 were essentially the same in wild type, *nb/+* and *nb/nb* mice, which indicates that the absence of ankyrin does not influence the expression of a key red cell membrane protein. In addition, the percentages of enucleated cells (Hoechst^{low}TER119^{high}) exhibited no significant differences in these mice (Figure 1A and 1B).

It is possible that stromal cells in the fetal liver compensate for the absence of ankyrin and facilitates the enucleation process. To circumvent this problem, we used our well-established *in vitro* erythroblast system in which purified E13.5 TER119 negative mouse fetal liver erythroblasts were cultured for 2 days on fibronectin- coated dishes in the presence of erythropoietin. The cells were harvested at day 2, stained with TER119 and Hoechst, and analyzed by flow cytometry. Figures 2A and 2B show that, as *in vivo*, loss of ankyrin did not affect the surface expression of TER119, or the extent of enucleation. These results demonstrate that ankyrin plays no significant function in enucleation of mammalian erythroblasts. These observations are also consistent with previously reported findings of no significant abnormalities of the red cell membrane or cytoskeleton in *nb/nb* mice [16].

We next investigated the role of band 3 in enucleation. To this end, we used targeted band 3 knockout mice. Figures 3A and 3B show that in contrast to *nb/nb* mice, the cell surface expression level of glycophorin A (TER119) is decreased significantly – about three-fold - in band 3 knockout mice both *in vivo* and in erythroblasts formed during *in vitro* culture. In addition, the band 3 null erythroblasts also exhibited abnormal Hoechst staining, which could also be due to the disrupted membrane/cytoskeleton network.

Deficiency of band 3 led to a slight increase of enucleation *in vivo* (Figure 3A upper panels and Figure 3C) compared to wild type and heterozygous mice. However, there were no significant changes in the extent of enucleation when the TER119 negative fetal liver erythroblasts were cultured *in vitro* (Figure 3A lower panels and Figure 3D). The minor increase of enucleated cells *in vivo* in band 3 knockout fetal liver is likely a reactive reticulocytosis in response to the hemolytic anemia in the mouse, consistent with findings from previous report [12].

4. Discussion

In summary, our study provides definitive evidence that ankyrin and band 3 are not required for mouse fetal liver erythroblast enucleation. The hypothesis that mammalian erythroid membrane and cytoskeleton proteins are involved in enucleation is based on the fact that chromatin condensation occurs in all vertebrates but that only mammalian erythroblasts undergo nuclear extrusion[2,17]. During erythropoiesis, the membrane and cytoskeleton proteins undergo extensive reorganization. At the end of erythropoiesis, spectrin, actin, tubulin, ankyrin, glycophorin A and protein 4.1 partition to the incipient reticulocytes, whereas no major erythroid membrane or cytoskeleton protein are found in the extruded nuclei [8,18,19,20]. One possibility is that these reorganizations may facilitate the enucleation process, since morphologic studies showed that during late stages of erythropoiesis the nucleus moves to a patch of membrane that is deficient in the spectrin network [18]. It is still unclear what triggers the movement of the nucleus and how the nucleus "decides" where to migrate. One possibility is that the nucleus moves specifically to a segment of the plasma membrane lacking ankyrin and/or band 3. However, the present results make it unlikely that ankyrin or band 3 are involved in this migration process.

The ankyrin-band 3 complex plays critical roles in bridging spectrin/actin cytoskeleton to the cell surface lipid membrane and serving as a docking site for multiple membrane/cytoskeleton proteins. Although no significant numbers of circulating nucleated red blood cells are present in ankyrin- or spectrin- deficient mice [11,12], enucleation could still have been compromised *in vitro* since various compensatory pathways could have rescued the enucleation defect *in vivo*. Therefore, the absence of any effect on the extent or efficiency of enucleation *in vitro* in the absence of these proteins is a surprising finding.

Besides the ankyrin-band 3 complexes, other complexes also maintain the integrity of the red blood cell bilayer membrane. These complexes, such as that containing α and β adducin, are also unlikely to play any role in enucleation given the very mild phenotype of β adducin null mice [21]. In contrast to mice with defects in these complexes that bridge the spectrin cytoskeleton with the plasma membrane, mice carrying spontaneous mutations of α-spectrin (*sph/sph* mice) or β-spectrin (*ja/ja* mice) exhibit severe hemolytic anemia and die within weeks of birth [13]. It would therefore be interesting to investigate the role of spectrins in enucleation using these *sph/sph* and *ja/ja* mice.

Acknowledgments

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Ankyrin and band 3 are not required for erythroblasts enucleation

Loss of ankyrin does not affect erythroid membrane glycoprotein expression

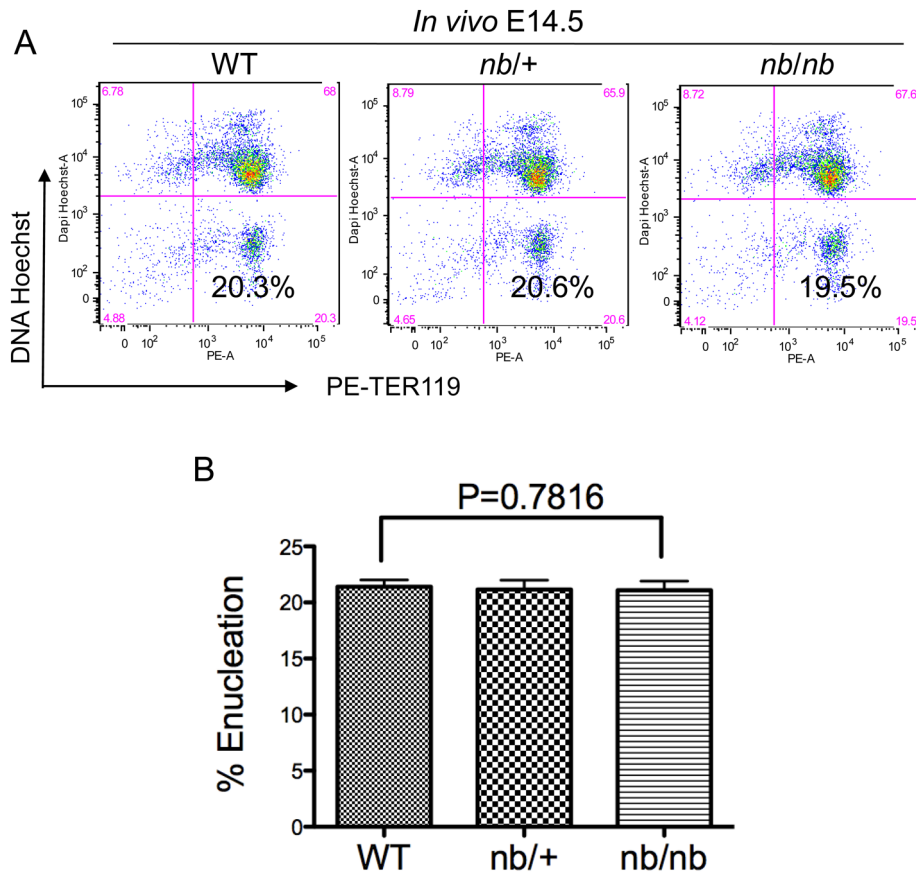Loss of band 3 influences erythroid membrane glycoprotein expression

Figure 1. Ankyrin deficiency does not affect cell surface TER119 expression or erythroblast enucleation *in vivo*

(A) Flow cytometric analysis of mouse fetal erythroblast enucleation *in vivo*. The total population of fetal liver cells from E14.5 embryos in wild type (WT), *nb/+*, or *nb/nb* mice was stained with Hoechst 33342 and PE conjugated TER119 and analyzed by flow cytometry. Debris (low forward scatter) and dead cells (propidium iodide positive) were excluded from the analysis. The percentage of enucleated cells is indicated in the lower right quadrant. (B) Statistical analysis of enucleation rate *in vivo* in wild type, *nb/+*, and *nb/nb* mice.
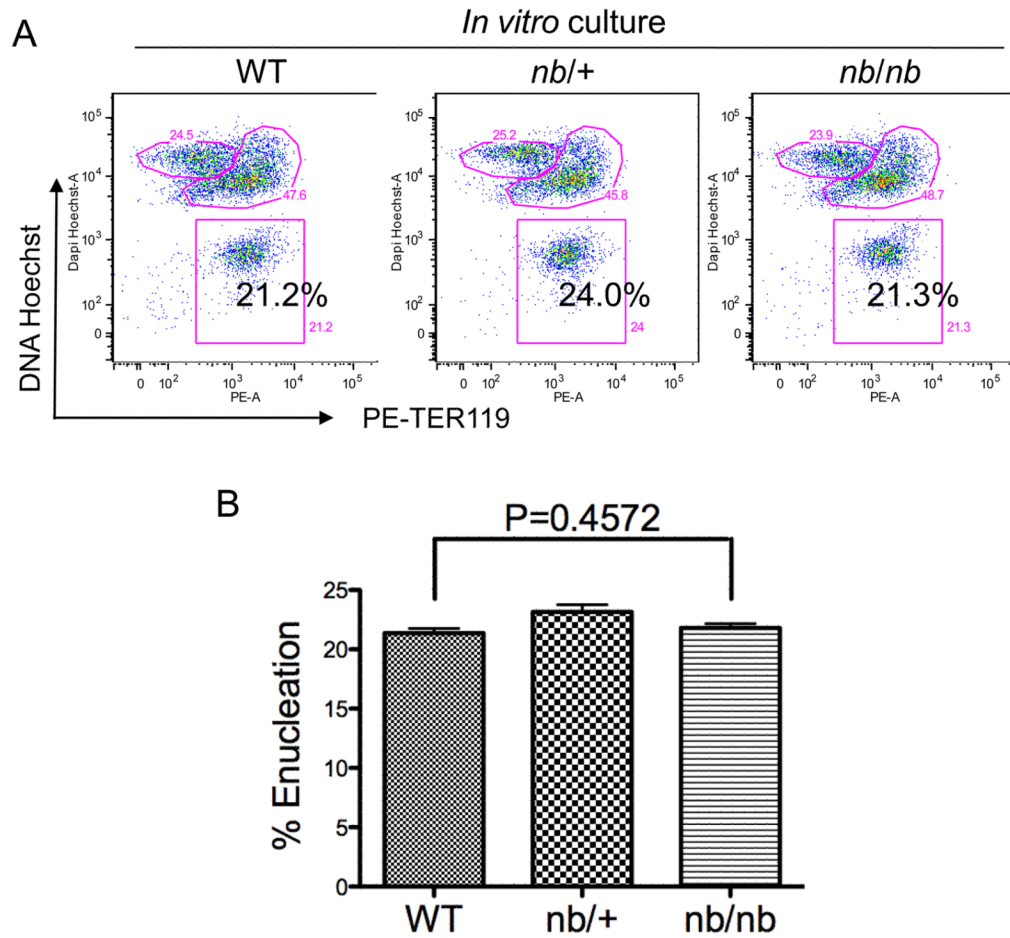
Figure 2. Ankyrin deficiency does not affect cell surface TER119 expression or erythroblast enucleation *in vitro*

(A) Analysis of mouse fetal erythroblast enucleation during *in vitro* culture. E13.5 fetal liver cells with the indicated genotype were stained with a biotinylated anti-TER119 monoclonal antibody. TER119 negative erythroid progenitor cells were purified and cultured *in vitro* in fibronectin-coated plates in medium containing serum and erythropoietin (Epo). The enucleation status of the cultured cells after two days of culture was analyzed by flow cytometric analysis using Hoechst 33342 and PE-TER-119. The percentage of enucleated cells is indicated. (B) Statistical analysis of the extent of enucleation *in vitro* in wild type, *nb/+*, and *nb/nb* mice.
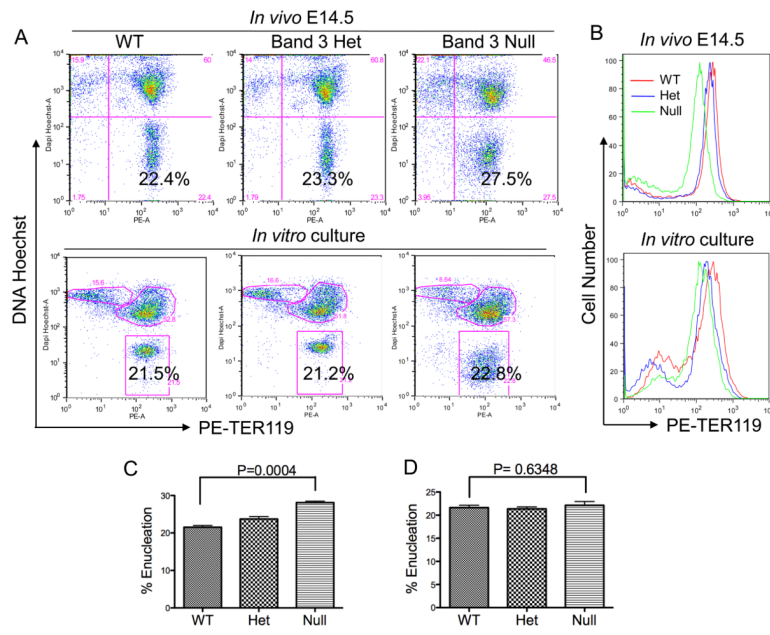
Figure 3. Absence of Band 3 reduces TER119 expression on the erythroblast cell surface but does not affect enucleation

(A) Flow cytometric analysis of mouse fetal erythroblast enucleation *in vivo*. The experiments were similar to those in Figure 1A (upper panels) and 2A (lower panels) except band 3 wild type (WT), heterozygous (Het), or knockout (null) mice were used. (B) Flow cytometric analysis of the surface TER119 expression levels of these mice *in vivo* (upper panel) and after in *vitro* culture (lower panel). Red, blue and green represent enucleated band 3 wild type (WT), heterozygous (Het) and knockout (null) erythroid cells, respectively. (C) Statistical analysis of enucleation extent *in vivo* in wild type, band 3 heterozygous and null mice. (D) Statistical analysis of enucleation extent *in vitro* in wild type, band 3 heterozygous and null mice.