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Protein phosphatase 2A regulatory subunit B56β modulates erythroid differentiation

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ABSTRACT

Anemia due to attenuated erythroid terminal differentiation is one of the most common hematological disorders occurring at all stages of life. We previously demonstrated that catalytic subunit α of protein phosphatase 2A (PP2Acα) modulates fetal liver erythropoiesis. However the corresponding PP2A regulatory subunit in this process remains unknown. In this study, we report that chemical inhibition of PP2A activity with okadaic acid impairs hemin-induced erythroid differentiation. Interestingly, B56 family member B56β is the only regulatory subunit whose expression is induced by both erythropoietin in fetal liver cells and hemin in erythroleukemia K562 cells. Finally, knockdown of B56β attenuates hemin-induced K562 erythroid differentiation. Collectively, our data identify B56β as the potential functional regulatory subunit of PP2A in erythroid differentiation, shedding light on new target for precise modulation of PP2A activity for treatment of anemia and related diseases.

1. Introduction

The production of mature erythroid cells from multipotent hematopoietic stem cells requires precise modulation of controlled cell proliferation, differentiation, survival, and death. Any blockage affecting turnover of mature red blood cells and survival of progenitor cells will lead to anemia [1]. Till now effective therapies still remain an unmet need and one of the hurdles is lack of complete understanding of the underlying mechanisms.

Reversible protein phosphorylation, mediated by protein kinases and phosphatases, represents the most common regulatory mechanism in eukaryotic cells [2]. As the major serine/threonine phosphatase [3], protein phosphatase 2A (PP2A) regulates multiple cellular processes including proliferation [4], metabolism, survival [5], migration [6], cell cycle [7], and differentiation [8].

PP2A core enzyme consists of a catalytic (PP2Ac) subunit and a structural scaffolding subunit (PR65/A). To get full activity, PP2A core enzyme recruits a third regulatory B subunit to form heterotrimers [9]. There are four regulatory subfamilies: B (PR55), B' (B56 or PR61), B" (PR72), and B‴ (PR93/PR110). These B subfamilies have diverse expression pattern and share low sequence similarity [10,11]. It is widely believed that distinct regulatory subunits recruit different substrates, thereby ensuring its temporal and spatial localization [12,13]. Recently, we demonstrated that conditional inactivation of its main catalytic subunit isoform PP2Acα in embryonic hematopoietic cells perturbed fetal liver erythropoiesis by promoting cell death of erythroid cells [5]. Nevertheless, the potential functional regulatory subunit(s) of PP2A in erythroid

Abbreviations: CFU-E, colony-forming unit-erythroid; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; GPA, glycophorin A; IMDM, Iscove's modified Dulbecco's medium; OA, okadaic acid; PCR, polymerase chain reaction; PP2A, protein phosphatase 2A; qRT-PCR, quantitative RT-PCR; 7-AAD, 7-aminoactinomycin D; CFU-E, colony-forming unit-erythroid; Pro-Es, pro-erythroblasts; Int-Es, intermediate; Late-Es, late erythroblasts.

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differentiation remains largely unknown, hampering precise modulation of its enzyme activity in anemia-related diseases.

Herein, we have identified the regulatory subunit B56β (also known as PPP2R5B, B'β, PR61B in mammals; pptr-1 in *C. elegans* [14]; Wbd in drosophila [15]) as the only regulatory subunit whose expression is stimulated by erythropoietin in fetal liver cells or by hemin in K562 cells. Based on the gene expression file data from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), we found that expression of B56β is much higher in erythroid progenitors (EP) than that in granulocyte/monocyte-macrophage progenitors (GMP) (GEO accession: GDS853) [16]. Besides, expression of B56β in human $CD34^+$ peripheral hematopoietic progenitor cells is gradually elevated during *in vitro* erythroid differentiation process (GEO accession: GDS2431) [17], as well as in enriched populations of colony-forming unit-erythroid (CFU-E), pro-erythroblasts (Pro-Es), intermediate (Int-Es), and late (Late-Es) erythroblasts which represent distinct differentiation stage (GEO accession: GDS3860) [18]. We demonstrated that knockdown of B56β decreases benzidine-positive cell percentage after hemin treatment. Collectively, our data characterize B56β as the corresponding PP2A regulatory subunit with physiological function in erythroid differentiation, shedding light on new target for precise modulation of PP2A enzyme activity for treatment of anemia.

2. Materials and methods

2.1. K562 cell culture and induced erythroid differentiation

Human chronic myelogenous leukemia k562 cells (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. Erythroid differentiation was induced with 50 μM hemin (Sigma) and assessed by benzidine test and expression of erythroid genes (α-globin, ε-globin and glycophorin A/GPA) as described previously [19]. K562 cells were cultured with 10 nM okadaic acid (OA, Sigma) for chemical inhibition of PP2A phosphatase activity as described previously [6].

2.2. Mice, timed mating and tissue harvesting

C57BL/6 mice were obtained from the Experimental Animal Center of the Chinese Academy of Medicine Sciences of Soochow University. All animal protocols were approved based on the local ethics legislation with respect to animal experimentation. Embryonic age was established by considering noon of the day that the vaginal plug was observed as embryonic (E) 0.5. Embryos were obtained at E15.5 after coitus and fetal livers were dissociated mechanically by pipetting. Single-cell suspensions were prepared as described previously [5]. Fetal liver cells were maintained in Iscove's modified Dulbecco's medium (IMDM) with 2% FBS for recombinant human erythropoietin (10 U/mL, Roche) stimulation.

2.3. Lentivirus vector construction and production

A mixture of two GFP expression lentiviral vectors which encode different short hairpin RNA (shRNA) sequences against B56β was purchased from Genechem (GV118, Shanghai, China). pSPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) were a gift from Didier Trono. For production of lentivirus, HEK293FT cells were cotransfected with GV118-shB56β, pSPAX2 and pMD2.G. Lentiviruses were harvested 72 h after transfection. After infection, GFP-positive K562 cells were sorted using a GFP positive cell sorter (Beckman MoFlo XDP).

2.4. Analysis of apoptotic cells

K562 cells were stained with anti-Annexin V & 7-aminoactinomycin D (7-AAD) according to the manufacturers' protocol (BD Pharmingen). Fluorescence activated cell sorting (FACS) analyses were performed using a Millipore Guava easyCyte as described previously [19].

2.5. Reverse transcription (RT)-PCR and quantitative (q) RT-PCR

RNA isolation and qRT-PCR analysis were performed as described previously [20,21]. Quantitative RT-PCR was performed using SYBR-RCR kit (Takara) on an ABI sequence detector (Stepone Plus, Applied Biosystems). Changes in the expression of target genes were calculated using $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (Ct_{target} - Ct_{GAPDH})_{sample} - (Ct_{target} - Ct_{GAPDH})_{control}$). Primer sequences are summarized in Table S1.

2.6. Statistical analysis

Data were analyzed with the two-tailed *t*-test and presented as mean ± SEM. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Hemin-induced K562 erythroid differentiation model

To induce erythroid differentiation, 50 μM hemin was added into culture medium of K562 cells for 5 days and benzidine test was used to stain mature erythroid cells. As illustrated in Fig. 1A, B, hemin treatment gradually elevates percentage of benzidine-positive cells from 6.90% to 58.2% ($P < 0.001$). Consistent with the above results, cell pellets after hemin treatment showed a bright red appearance compared with that before hemin treatment (Fig. 1C). Besides, erythroid genes α-globin, ε-globin and GPA are all substantially induced by 50 μM hemin stimulation accordingly ($P < 0.01$ for α-globin; $P < 0.05$ for ε-globin; $P < 0.05$ for GPA) (Fig. 1D).

3.2. Okadaic acid inhibits hemin-induced erythroid differentiation

We next examined how PP2A enzyme activity modulates erythroid differentiation. Potent inhibition of endogenous PP2A activity was achieved by treating K562 cells with okadaic acid (OA), which inhibits PP2A enzyme in a dose- and time-dependent manner. OA was added into the K562 erythroid differentiation system at the final concentration of 10 nM as described previously [6]. OA treatment decreases percentage of benzidine-positive cells by 31.8% compared with hemin group ($P < 0.001$), indicating inhibition of PP2A activity attenuates K562 erythroid differentiation (Fig. 2A, B). Cell pellet appearance of the three groups is in accordance with benzidine staining results (Fig. 2C). Besides, OA also elevates apoptosis of K562 cells during hemin treatment (2.08-fold for early apoptosis (Annexin V^+/7-AAD^-); 1.36-fold for late apoptosis (Annexin V^+/7-AAD^+)) (Fig. 2D).

3.3. Expression of B56β is induced during erythroid differentiation

Fetal liver erythropoiesis is considered to be mechanistically similar to adult stress erythropoiesis, as both processes are erythropoietin (EPO) responsive. To elucidate the potential functional regulatory subunit(s) in erythropoiesis, we examined their expression pattern after EPO treatment in primary fetal liver cells. As illustrated in Fig. 3A, B56β is the only regulatory subunit whose

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