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Differential gene expression profiling between wild-type and ALAS2-null erythroblasts: Identification of novel heme-regulated genes ☆

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Abstract

To identify erythroid-specific heme-regulated genes, we performed differential expression analysis between wild-type and heme-deficient erythroblasts, which had been prepared from wild-type and erythroid-specific δ-aminolevulinate synthase-null mouse ES cells, respectively. Among 8737 clones on cDNA array, 40 cDNA clones, including 34 unknown ESTs, were first selected by their high expression profiles in wild-type erythroblasts, and evaluated further for their erythroid-lineage specificity, expression in hematopoietic tissues in vivo, and heme-dependent expression, which yielded 11, 4, and 4 genes, respectively. Because of the selection strategy employed, the final 4 were considered as the newly identified erythroid-specific heme-regulated genes. These 4 genes were uncoupling protein 2, nucleolar spindle-associated protein, cellular nucleic acid-binding protein, and a novel acetyltransferase-like protein. These findings thus suggest that heme may regulate a wide variety of hitherto unrecognized genes, and further analysis of these genes may clarify their role in erythroid cell differentiation.

Keywords: Heme; cDNA array; Gene; ALAS2; Erythropoiesis

Erythroid cells synthesize and accumulate an enormous amount of hemoglobin in the late stage of their differentiation. Enhanced synthesis of hemoglobin during erythroid cell differentiation requires a coordinated expression of genes involved in heme biosynthesis, globin formation, and iron transport [1]. These cells also turn on a set of anti-oxidant genes which are important in erythroid maturation [2]. Conversely, erythroid cells turn off a large number of non-erythroid genes along their cell differentiation. However, the exact mechanism that governs the complex erythroid program remains unclear, since many genes involved in this process have not yet been defined.

Heme is essential for the function of all aerobic cells. Besides its role as the prosthetic group of hemoproteins, heme is known to regulate gene expression of several genes

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in eukaryotic cells [3–5]. This aspect is particularly important to consider in erythroid cells, since ≈85% of heme in the body is synthesized by erythroid cells and utilized for hemoglobin formation [1]. It is therefore possible that, in addition to hemoglobin synthesis, heme may also play another important role in the erythroid cell differentiation program.

The first and the regulatory step of heme biosynthesis in erythroid cells is catalyzed by the erythroid-specific δ-aminolevulinate synthase, ALAS2 (or ALAS-E) [6,7]. Recently, we established ALAS2-null mouse embryonic stem (ES) cells and successfully generated heme-deficient definitive erythroblasts from these mutant ES cells in vitro [8,9]. In this study, we compared the gene expression profile between wild-type and heme-deficient erythroblasts, which were generated in an in vitro ES cell culture system, to identify novel heme-regulated erythroid-specific genes. We focused on the genes which had: (i) higher expression in wild-type than in ALAS2-null erythroblasts, (ii) erythroid-lineage specificity (iii) high expression in hematopoietic tissues in vivo, and (iv) heme-dependent expression. This strategy has led us to identify 4 heme-regulated erythroid-specific genes, which may play an important role in erythroid differentiation.

Materials and methods

cDNA array. ALAS2-null and wild-type definitive erythroblasts were prepared from ALAS2-null and wild-type mouse ES cells, respectively, as described previously [9,10]. Gene expression profile analysis was performed by using a cDNA array on which 8737 cDNA fragments (Aligent Technologies, Palo Alto, CA) were aligned. To obtain the full-length cDNA of unknown genes, a 5′-RACE Kit (GeneRacer Kit; Invitrogen, Carlsbad, CA) was used. All PCR products were ligated to the pGEM-T Easy cloning vector (Promega, Madison, WI) and sequenced subsequently.

Cell sorting. Mouse bone marrow cells were separated by a magnetic cell sorting system (MACS; Miltenyi Biotec, Gladbach, Germany) using the following antibodies for representative hematopoietic lineages: TER119 (Pharmingen, San Diego, CA), an antibody against the mature erythroid-specific antigen; CD11b (Caltag Laboratories, Burlingame, CA), an antibody against the granulocyte-specific antigen; CD3 (Caltag Laboratories), an antibody against the T cell-specific antigen; B220 (Pharmingen), an antibody against the B cell-specific antigen; and Sca1 (Pharmingen), an antibody against the stem cell-specific antigen. Cytospin cell smear preparations were made on glass slides, and stained with May–Giemsa stain.

Extraction of mouse tissue RNA. Mouse brain, lung, heart, liver, spleen, kidney, and gut were frozen in liquid nitrogen immediately after isolation of the tissue. Frozen tissues were homogenized and total RNA was obtained using ISOGEN (Nippon Gene, Tokyo, Japan). Bone marrow cells were flushed out from tibiae and femur, and total RNA was extracted using ISOGEN-LS (Nippon Gene, Tokyo, Japan).

Northern blot and quantitative RT-PCR analysis. For Northern blot analysis, total RNA (20 μg/lane) was loaded onto each lane in a 1% formaldehyde–agarose gel, electrophoresed in MOPS buffer, and transferred to Hybond-N^+ nylon membranes (Amersham–Pharmacia Biotech, Piscataway, NJ). cDNAs were labeled with [α-^{32}P]dCTP by the random priming method (Rediprime II Random Prime Labeling System; Amersham Biosciences, UK) and were used as the hybridization probe. Radioactive signals were detected by exposing filters to X-ray films (Bio-Max XAR, Eastman Kodac, Rochester, NY).

RNA of the candidate genes was also reverse-transcribed using SuperScript (Invitrogen) and subjected to quantitative RT-PCR using Quantitect SYBR green PCR Kit (Qiagen, GmbH, Germany). Primers for quantitative RT-PCR were designed from DNA sequences based on their GenBank accession number (http://www.ncbi.nlm.nih.gov/). The amount of each gene transcript was normalized based on the level of GAPDH mRNA which was included as reference.

Generation of erythroblasts with various amounts of heme. Erythroblasts that contain various amounts of heme were generated by the in vitro ES cell differentiation system. First, partially rescued ALAS2-null ES subclones were prepared from ALAS2-null mouse ES cells by transgenic expression of human ALAS2 cDNA which was driven by GATA-1, the erythroid-specific promoter. Briefly, human ALAS2 cDNA was inserted downstream of GATA-1 promoter [11] and electroporated to ALAS2-null mouse ES cells with a hygromycin-resistant vector (BD Clontech, Palo Alto, CA). These cells were cultivated in ES medium containing 400 μg/ml of hygromycin. Hygromycin-resistant cells were cloned and transgene expression was confirmed by PCR. Then, both wild-type and human ALAS2-transgenic ALAS2-null mouse ES cells were subjected to undergo erythroid differentiation in culture as described previously [9,10], and definitive erythroblasts were collected on day 14. In this system, transgenic human ALAS2 was expressed under the control of the GATA-1 promoter when cells underwent erythroid differentiation, and various amounts of heme were found in the cell, which reflected various levels of expressed human ALAS2 expression.

Heme content. Heme content was determined fluorometrically as described previously [6].

Results

cDNA array

We picked genes that showed at least 3 times greater expression in wild-type erythroid cells compared with heme-deficient erythroid cells. As the level of expression of these genes ranged widely, we decided to deal with the top 50% highly expressed genes, but to discard the bottom 50% as it seemed extremely difficult to detect them in vivo, because of their low expression in the differential array. This step yielded 120 candidate genes that had a significant level of expression, as well as a markedly higher expression in wild-type erythroblasts compared with heme-deficient erythroblasts. Among the 120 genes, 34 genes were unknown ESTs. In contrast, 86 genes were already known, which included heme-oxygenase 1 (HO-1) [5] that was known to function in a heme-regulated manner. In this study, we focused on 40 genes which included all of the 34 unknown ESTs and 6 known genes but whose role in erythropoiesis has not been defined.

Erythroid-lineage-specific expression

First, the specific primers for these 40 genes were prepared and the expression in specific hematopoietic lineage cells was examined by quantitative PCR. Among the 40 genes, 11 genes (Table 1) were considered to be erythroid-specific, as they showed an almost exclusive expression in TER119-positive cells (Fig. 1). Among them, 5 genes, i.e., UCP2, NuSAP, CNBP, protein kinase N2, and 2′5′-oligoadenylate synthase, had previously been described, but their role in erythroid differentiation has

Table 1
Erythroid-lineage specific genes

BC012697	Uncoupling protein 2 (UCP2)
AA920753	Nucleolar spindle-associated protein (NuSAP)
AA065512	Cellular nucleic acid-binding protein (CNBP)
AI173087	EST1 (acetyltransferase-like)
AA691164	Protein kinase N2 (PRK2)
AA123007	2′5′-Oligoadenylate synthase (Oasl2)
AI608071	EST2 (protein phosphatase-like)
AA734249	EST3
AI414696	EST4
AA619953	EST5
AI180925	EST6

not been examined. Remaining 6 genes have not been reported or characterized. Database search of these six unknown EST clones demonstrated that full-length cDNA sequence of two of them, EST1 and EST2, might be compatible with an acetyltransferase and a protein phosphatase, respectively.

Expression in hematopoietic tissues in vivo

We examined tissue-specific expression of the 11 erythroid-lineage specific genes by Northern blot analysis and found that 4 genes, NuSAP, UCP2, CNBP, and EST1, were expressed significantly in bone marrow (Fig. 2), while 7 others did not (data not shown). Expression of UCP2 had previously been reported in lung and in spleen [12], and our observation was also consistent with the previous finding. NuSAP mRNA was almost exclusively expressed in bone marrow (Fig. 2). CNBP mRNA was universally expressed in various organs, e.g., bone marrow, lung, brain, and kidney (Fig. 2). EST1 mRNA was expressed in gut, kidney, and bone marrow at a similar level. These findings thus demonstrate that, among the 11 erythroid-lineage specific genes, NuSAP, UCP2, CNBP, and EST1, are also expressed in vivo in hematopoietic tissues such as bone marrow and suggest that they may have a potential role in erythropoiesis.

Heme-dependent gene expression

Next, we examined heme-dependent gene expression of these 4 genes. For this purpose, we used wild-type erythroblasts which contained significant amounts of hemoglobin upon cell differentiation, and ALAS2-null erythroblasts which are practically heme-deficient. In addition, we prepared erythroblasts which contained various amounts of heme, derived from ALAS2-null ES cells which were partially rescued by transgenic expression of human ALAS2 cDNA. In this system, heme was synthesized intracellularly from ALA that was provided by the transgenic expression of human ALAS2. Thus, this system resembles endogenous heme biosynthesis in normal erythroblasts and is thought to be more natural than the exogenous supply of hemin to culture medium. The cell pellet and heme content of erythroblasts derived from ES cells of wild-type, ALAS2-

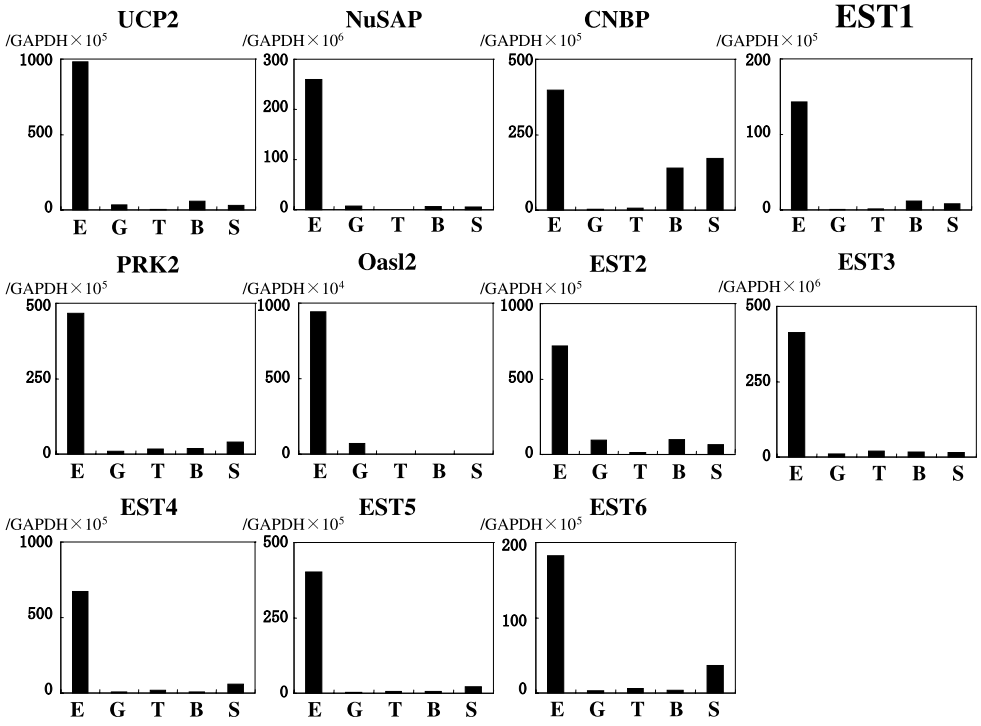

Fig. 1. Erythroid-lineage specific expression of the candidate genes. E, G, T, B, and S represent erythroid, granulocytic, T cell, B cell, and stem cell lineage, respectively. The level of mRNA expression was corrected based on the level of GAPDH mRNA. Data are representative of two independent experiments. Results for the 11 genes that showed erythroid-lineage specific expression are shown.

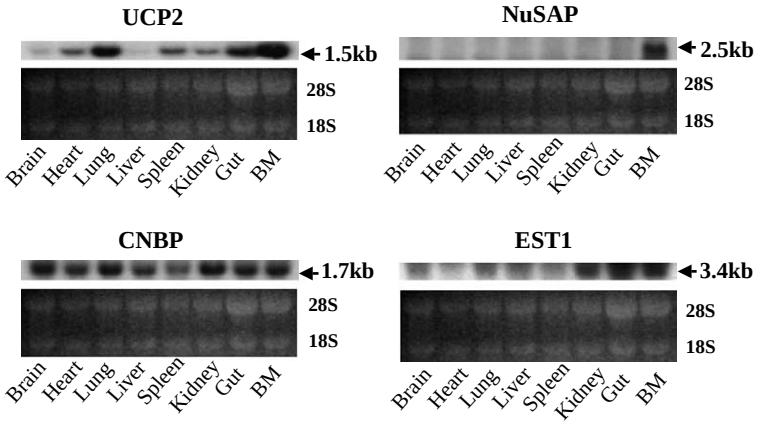

Fig. 2. UCP2, NuSAP, CNBP, and EST1 mRNA expression in vivo. Northern blot analysis was performed for mRNAs for 11 erythroid-lineage specific genes using various mouse tissues. The results for 4 clones that showed hematopoietic tissue abundant expression, i.e., UCP2, NuSAP, CNBP, and EST1, are shown, while the remaining 7 clones did not express significantly in hematopoietic tissues (data not shown). Total RNA was isolated from mouse brain, heart, lung, liver, spleen, kidney, gut, and bone marrow, and used for analysis for mRNA expression. cDNA probes were labeled with [α-^{32}P]dCTP by the random priming method as described in Materials and methods, and were used as the hybridization probe.

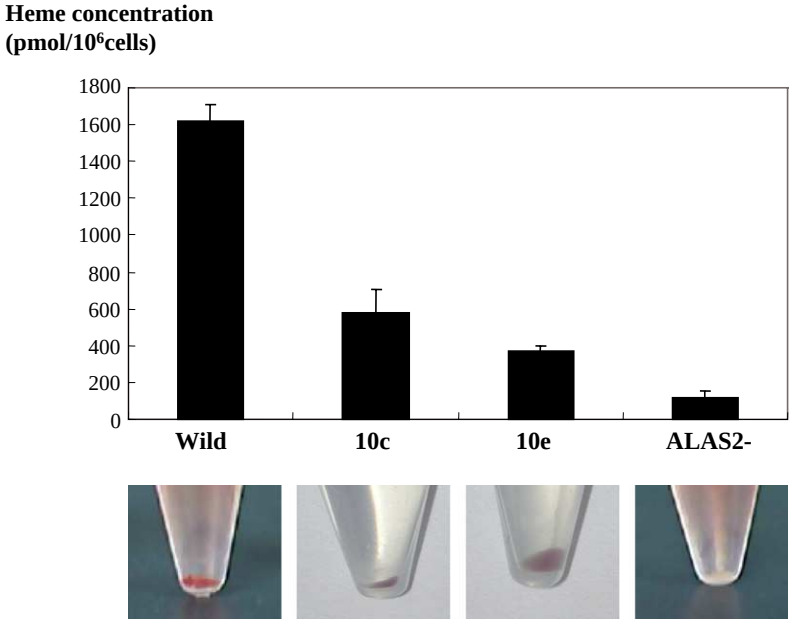

Fig. 3. Heme content and the view of clones rescued from ALAS2-null ES cells by transgenic expression of human ALAS2. Heme concentration of wild-type, heme-deficient (ALAS2-null), and two rescued clones, 10c and 10e, is shown in the upper inset. Cell pellets of these cells are shown in the lower inset.

null, and clones 10c and 10e, two representative clones rescued by human ALAS2 transgene expression, are shown in Fig. 3. As reflected in their red color, hemoglobinization was most intense in wild-type erythroblasts, followed by clone 10c, clone 10e, while ALAS2-null cells which were hardly hemoglobinized. Determination of heme content in these cells also confirmed the same trend. The level of expression of the 4 candidate genes (Fig. 2) was also evaluated in the same series of erythroid cells. The results showed that all of the 4 genes showed heme-dependent expression (Fig. 4). These results indicate that the level of the expression of the 4 genes was determined by intracellular heme concentration, which in turn reflected on the level of human ALAS2 expression.

Characterization of EST1

Based on database search, EST1 was found to share a complete identity with the genomic sequence, BC038816 (GenBank Accession No.), which spans approximately 3.4 kb, comprises of three exons and two introns, and maps to mouse chromosome 11q2. The mouse EST1 consists of 110 amino acids (Fig. 5), which is 100% identical to the rat (GenBank Accession No. XP343908), 92.7% to the human (Accession No. NP690878), and 90.9% to the chicken gene (Accession No. CAG32107). Western blot analysis using a His-tagged EST1 demonstrated that a molecular weight of mouse EST1 is $\approx$12 kDa (data not shown). EST1 may belong to the GNAT (GCN5-related *N*-acetyl-

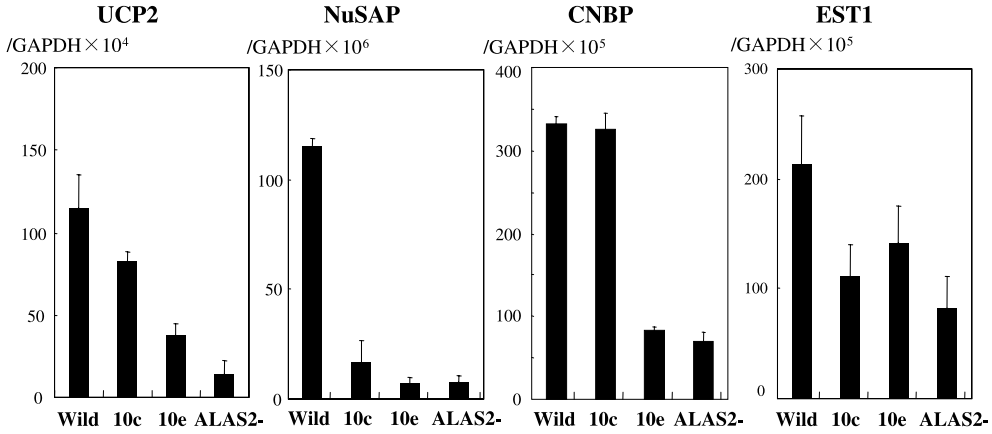

Fig. 4. Heme-dependent expression of 4 candidate genes. Quantitative RT-PCR analysis was performed using wild, clones 10c, 10e, and ALAS2-null erythroblasts for the 4 candidate genes. The results for 4 clones that showed heme-dependent gene expression, i.e., UCP2, NuSAP, CNBP, and EST1, are shown. The level of mRNA expression was corrected based on the level of GAPDH mRNA. Data are means ± SD of three separate experiments.

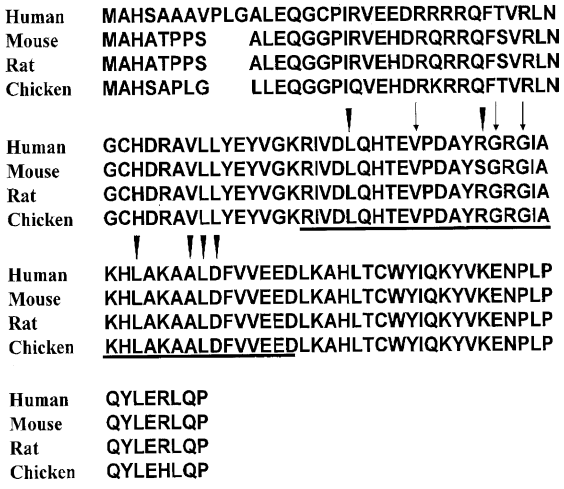

Fig. 5. Amino acid sequence of EST1. As shown, the sequence encodes 110 amino acids. The conserved *N*-acetyltransferase domain is underlined; highly and moderately conserved amino acid residues are indicated by arrow and arrowhead, respectively [13].

transferase) superfamily, as it shows the similarity to the *N*-acetyltransferase domain, containing several conserved amino acid residues (single-underlined in Fig. 5), which is also known as the putative acetyl CoA-binding domain [13].

Discussion

In this study, we sought to identify novel erythroid-specific heme-regulated genes. For this purpose, both the heme-deficient and heme-containing erythroblasts were prepared in an in vitro ES cell differentiation system. We have reported that erythroblasts generated from wild-type ES cells in this system were quite similar to in vivo erythroblasts judging from their phenotype, whereas erythroblasts generated from ALAS2-null ES cells were heme-deficient in spite of their normal morphology [9]. In addition, we established ALAS2-null ES subclones which were partially rescued by transgenic human ALAS2 expression to generate erythroblasts which contain various amounts of heme. In erythroblasts induced from these subclones, heme was supplied by the endogenous heme synthesis pathway involving transgenic human ALAS2; therefore, the effect of heme content on gene expression could be examined in a more physiological condition compared to the exogenous supply of hemin. Thus, we believe that our in vitro system could faithfully reproduce the in vivo condition and is suitable for identification of in vivo heme-regulated genes.

We studied the difference in the gene expression of heme-deficient erythroblasts generated from ALAS2-null ES cells compared with heme-containing wild-type ES cells. This approach yielded 120 genes which showed significant expression in wild-type than in heme-deficient erythroblasts. Among the 120 genes, 34 unknown ESTs, and 6 known genes were further evaluated for their erythroid-lineage specificity, expression in hematopoietic tissues in vivo in mice and heme-dependent expression. Ultimately 4 genes, UCP2, NuSAP, CNBP, and EST1, were identified as novel erythroid-specific heme-regulated candidate genes. Among them, 3 known genes, UCP2, NuSAP, and CNBP, have been reported to be involved in the protection against reactive oxygen species [12], cell cycle regulation [14], and cell growth [15], respectively. Since erythroid cells are regularly exposed to reactive oxygen species generated by high concentration of iron, a protection system is essential to complete their maturation without any cellular damage. In addition, the cell cycle and growth must be tightly regulated to supply red blood cells which are renewed continuously and to respond to an emergent blood loss. Thus, heme regulates a wide variety of genes which may be involved in fundamental functions for maintenance of normal erythropoiesis. On the other hand, EST1 was proved to be a novel heme-regulated acetyl-CoA-binding protein (Fig. 5), which might belong to the GNAT superfamily [13]. Although the functional significance of EST1 remains uncertain, this novel protein should offer opportunities for further experimentation.

In conclusion, our present study has demonstrated the identification of 4 heme-regulated erythroid-specific genes and also suggested that heme may play a critical role in regulating various gene expressions in erythroid cells that have not been known to date. Elucidation of the function and the role of these candidate genes in the erythroid program may shed light on the pathophysiology of anemia due to heme deficiency, e.g., sideroblastic anemia, or refractory anemia.

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