should expressed from targeted allele instead of ALAS-N protein. Since targeted allele derived GFP mRNA does not fused to Alas1 mRNA, which stability was regulated by intracellular heme level (see above), expression level of GFP should mimic the transcriptional level of Alas1. In this context, authors have tried to determine the cell specific or tissue specific transcriptional regulation of Alas1 gene using heterozygous ($Alas1^{+/GFP}$) mice.

In adult $Alas1^{+/GFP}$ mice, observation using fluorescent microscopy revealed that GFP fluorescence was observed in all tissues examined, and the strong GFP fluorescence was observed in liver, Harderian glands, testes (Leydig cells and spermatid), exocrine glands (submandibular and parotid glands) and endocrine glands (adrenal and thyroid glands), hematopoietic tissues (myeloid cells) and brain (meninges and choroid plexus). Remarkable GFP fluorescence was also observed in mucous epithelium cells in oral cavity, pharynx, larynx, nasal cavity and respiratory system (trachea and bronchi). These results suggested that intensity of GFP fluorescence seemed to correlate to demand for heme supply for drug metabolism (liver and lung) and steroid hormone production (Leydig cells and adrenal gland). Interestingly, strongest GFP fluorescence among hematopoietic cells was observed in granulocytes, in which myeloperoxidase was expressed as a hemoprotein[85].

Using $Alas1^{+/GFP}$ mice, authors also examined whether ALAS-N expression in liver oscillated or not, in a 12 hour light and 12 hour dark cycle. As a result, it was confirmed ALAS-N mRNA expression, as well as GFP mRNA expression, displayed similar circadian oscillation[84], suggesting that basic circadian control for ALAS-N mRNA expression might be regulated at transcriptional level.

VII. Regulation of ALAS-E (ALAS2) expression

Expression of ALAS-E is restricted in erythroid cells, in which large amount of heme is produced for hemoglobin formation. Therefore apparent negative feedback regulation for ALAS-E expression by heme was not

observed. Instead, erythroid cells are able to export excess amount of heme from cytosol to the outside through feline leukemia virus subgroup C receptor (FLVCR), and suppression of FLVCR in K562 cells impaired erythroid maturation and leades to apoptosis[86]. Moreover, genetic ablation of FLVCR gene causes lack of definitive erythropoiesis and FLVCR-null mice die in midgestation[87], suggesting that erythroid progenitor cells hardly regulate heme production precisely during its terminal differentiation.

A. Transcriptional regulation of ALAS-E gene (ALAS2)

1. Identification of several erythroid specific cis acting elements in the proximal promoter region of mouse Alas2 gene

Cloning of mouse ALAS-E gene was first reported by Schoenhaut and Curtis in 1989[20]. Mouse Alas2 gene consists of eleven exons, and spans about 24 kbp. These authors identified five independent erythroid specific DNAse I hypersensitive sites, one of which located at promoter region. Moreover, putative SP-1 binding site was recognized at proximal promoter region, although functional consequence of this cis element remained unknown[20].

Functional analysis of mouse Alas2 promoter was intensively performed by Kramer et al.[88]. These authors first cloned genome DNA fragment of Alas2 gene, which contained promoter region including up to 1.4 kbp upstream of transcription initiation site. Several deletion mutants were prepared to determine the essential region for transcriptional activity. As a result, they found that proximal -714 bp promoter region required for full promoter activity in erythroid cells, as well as in non-erythroid cells. Within this region, authors identified several putative cis-acting elements important for erythroid differentiation, including EKLF/Sp1, two GATA-1 and NF-E2 and HIF-1. Further deletion of promoter region revealed that a region located between -538 to -315 bp was required for transcriptional activation of Alas2 during erythroid differentiation, however, no known erythroid-specific cis element was identified in this region except for HIF-1 binding site. Authors thus proposed that unknown erythroid-specific

regulatory element(s) might be located within this region. Furthermore, authors confirmed that GATA-1 protein could bind to both GATA-1 binding sites, and disruption of either GATA-1 binding site in -714 bp promoter fragment decreased its promoter activity in erythroid cells to about 50%, while such modification of promoter sequence did not affect its promoter activity in non-erythroid cell. These results suggest that both GATA-1 binding sites in proximal promoter region are critical to confer erythroid-specific expression of mouse Alas2 gene[88].

2. Transcriptional regulation of human ALAS2 gene by its promoter

Cox et al. first reported the proximal promoter sequence of human ALAS2 gene, and identified several erythroid-specific transcription factor binding site, such as GATA, CCAAT and NF-E2, as well as TATA sequence within 100bp upstream of transcription initiation site[89].

Surinya et al. also cloned human ALAS2 promoter region including 10.3 kbp upstream of transcription initiation site, and tried to determine an important region for erythroid-specific expression of ALAS2[90]. Based on promoter deletion assay, authors found that proximal 293 bp of ALAS2 promoter exhibit the strongest promoter activity in erythroid cells. Within this region, authors identified several erythroid specific cis-elements, including GATA, Ets, CCAAT, CACCC and NF-E2, and they tried to examine biological significance of GATA, CACCC and NF-E2 consensus sequence. There were three putative GATA binding sites within 140 bp from transcription initiation site, two of which were relatively distal region (located at -124 and -100 bp from transcription initiation site, named as distal GATAs) and the other one was located at proximal region (named as proximal GATA). It was confirmed that GATA-1 could bind to these three GATA sequence by gel-retardation assay, and promoter assay experiments revealed that all these GATA sequence were functional in erythroid cells. Interestingly, proximal GATA sequence was partially overlapped with TATA-like sequence, at which association of TATA-binding protein (TBP) was confirmed. Since the conversion of this overlapped sequence to canonical

GATA binding sequence or canonical TATA sequence resulted in the reduction of promoter activity, authors suggested that functional interaction of GATA-1 and TBP at this element might be important for maintaining appropriate promoter function. Authors also demonstrated that CACCC sequence was functional in erythroid cells by binding erythroid Krüppel-like factor. On the other hand, these authors concluded that NF-E2 sequence was not functional on this promoter, and it is consistent with the result shown by Andrews et al[91], in which NF-E2 element in human ALAS2 proxymal promoter did not bind NF-E2 transcription factor.

Bekri et al.[92] identified a mutation at 206 bp upstream of transcription initiation site in patient with X-linked sideroblastic anemia, which was caused by the loss of function mutation of ALAS2 gene[93]. Authors found that ALAS2 mRNA level in patient's erythroid precursors was decreased to 87% of normal subject, and confirmed this promoter mutation reduced the promoter activity in K562 erythroid cells, whereas it did not affect promoter activity in non-erythroid cells. Barton et al. also reported that this mutation might be related to the severe iron overload observed in patients with sickle cell anemia[94]. However, biological impact of this promoter mutation remained to be elusive, since this mutation was also reported as SNP[95].

3. Erythroid-specific enhancer in intron 8 for transcriptional regulation of ALAS2 gene

Surinya et al.[96] searched the erythroid-specific enhancer throughout human ALAS2 gene by sequencing analysis of whole gene, and compared it with the previously reported DNAse I hyper sensitive sites in mouse Alas2 gene[20]. As a result, authors identified three independent putative enhancer regions at intron 1 (4.9 kbp), intron 3 (850 bp) and intron 8 (480 bp). Using transient transfection of reporter plasmids into erythroid and non-erythroid cells, authors found that intron 1 and intron 8 could enhance ALAS2 proxymal promoter activity, whereas intron 3 showed rather inhibitory effect. Further analysis using intron 8, in which two CACCC elements and four GATA binding elements were identified, revealed that this region

enhanced ALAS2 proxymal promoter activity to twelve times higher level in K562 human erythroid cell lines. Importantly, such enhancing effect of this region was not observed in non-erythroid cell line, suggesting that this enhancer functioned in erythroid-specific manner. Deletion analysis of intron 8 further revealed that both CACCC elements and two GATA binding elements (adjacent to CACCC elements) played a critical role in K562 cells. These two CACCC elements (designated CACC-A and CACC-B) and two GATA elements (designated GATA-A and GATA-B) were lined from 5' to 3' of coding strand, and the disruption of any cis element, except for GATA-A disruption, diminished the enhancing activity of this region. Using nuclear extract from erythroid cell, authors confirmed that GATA-1 protein bound to GATA elements, and SP-1 protein and other unknown protein bound to CACCC elements. However, ectopic expression of GATA-1 alone or in combination of GATA-1 and SP-1 could not accelerate enhancing activity of this region, suggesting that binding of unknown protein on CACCC elements might critical for erythroid-specific enhancing activity of this region[96].

4. Hypoxic upregulation of ALAS2 mRNA expression

Hypoxia induces several proteins involved in erythropoiesis, such as erythropoietin[97], transferrin[98] and transferrin receptor[99], by activating hypoxia inducible factor 1α (HIF-1α), which bind to hypoxia responsive element (HRE) as a heterodimer with HIF-1β[100]. Since putative HRE was identified in the proximal promoter region of murine Alas2 gene[88], Hofer et al. examined the effect of hypoxia on the expression level of ALAS2 mRNA in murine erythroid cells[101]. As a result, hypoxic upregulation of Alas2 mRNA was confirmed, and responsible promoter region of ALAS2 gene for this hypoxic upregulation was identified. Although this promoter region contained putative HRE, further experiments revealed that hypoxic induction of murine ALAS2 was independent of this putative HRE. Thus, responsible mechanism for hypoxic upregulation of ALAS2 gene still remains elusive.

In human, Narayan et al. reported that hypoxia induced erythroid differentiation of hematopoietic stem cell (CD34+/Lin-) in vitro, which was related to the induction of ALAS2 mRNA[102]. Kaneko et al. also reported increased hemoglobinization of human erythroleukemia cells along with the induction of ALAS2 mRNA in these cells[103]. Authors tried to determine the involvement of HIF-1α in ALAS2 upregulation, however, a line of evidence indicated that HIF-1α did not directly regulate ALAS2 transcription in these cells.

On the other hand, Zhang et al identified three putative HRE, which were clustered at the 3' flanking region of human ALAS2 gene[104]. Authors showed that hypoxia induced ALAS2 mRNA in normal erythroid progenitor cells derived from $CD34^+$ stem cells, as well as in K562 human erythroid cells. Then it was confirmed that the DNA fragment included these putative HREs (located between +610 and +750 from the 3' end of the last exon of ALAS2 gene) enhanced SV40 promoter activity in non-erythroid cells under hypoxic condition. Moreover, direct binding of HIF-1α to this region in vivo was demonstrated using chromatin immunoprecipitation assay (ChIP), suggesting that these HREs were functional[104]. However, it should be noticed that the 3' flanking region of ALAS2 gene is overlapped with apurinic/apyrimidinic endonuclease 2 gene (APEX2), and indicated region for putative HREs is located at the last exon of APEX2 gene (accession: NG_008983). Thus, it should be carefully examined whether this HRE was involved in the hypoxic regulation of human ALAS2 gene.

B. Translational regulation of ALAS-E

1. Intracellular iron regulates ALAS-E translation

It has been established that iron regulatory protein (IRP) bind to the stem loop structure of mRNA (referred as iron responsive element, IRE) to regulate the translation of candidate mRNA[105]. IRE has been identified at 5'- or 3'- untranslated region (UTR) of several mRNAs, which encode proteins involving iron metabolism, such as ferritin[106], transferrin receptor 1 (TFR1)[107], divalent metal transporter 1 (DMT1)[108], mitochondrial

aconitase[109], ferroportin[110] and ALAS2[89, 111]. IRP is able to bind to IRE, when intracellular iron concentration is low. The binding of IRP to IRE exerts the differential effects on translation of the target mRNAs, depending on the position of IRE on each mRNA. When IRP binds IRE at 5'-UTR region (ferritin, mitochondrial aconitase, ferroportin and ALAS2), IRP prevents the binding of translation initiation complex to mRNA, thereby inhibiting the translation of target genes[112]. On the other hand, mRNA is protected from the degradation mediated by RNase, leading to increased translation, when IRE binds IRP at 3'-UTR region (TFR1 and DMT-1)[113]. Thus, expression of TFR1 and DMT-1 protein are increased to import iron from outside the cells into cytoplasm, whereas expression of ferritin protein and ferroportin level is decreased to keep utilizable iron in cytosol during iron deficiency condition.

IRPs consist of two structurally related proteins, IRP1 and IRP2. These two proteins share 79% similarity in amino acid sequence, and both proteins bind tightly to IREs. However, the regulation of these proteins are quite different[114]. IRP1 is a stable bifunctional protein, which functions either as a cytosolic aconitase or as an IRP1, in the presence or absence of iron sulfur cluster, respectively[115]. In contrast, IRP2 expression is regulated in iron-replete cells by its oxidation-induced ubiquitination and subsequent degradation by the proteasome[116]. Using knockout mice of each IRP, Meyron-Holtz et al. have revealed that IRP2 dominates the regulation of iron homeostasis in vivo[117].

In erythroid cells, however, TFR1 expression seems to be distinctively regulated from other cells during erythroid maturation. For example, Chen et al. reported that the stability of TFR1 mRNA was increased in differentiating mouse erythroid cell line even under the iron overloaded condition, suggesting that TFR1 mRNA level was maintained at transcriptional level in erythroid cells[118]. In fact, Lok and Ponka found that proximal promoter region of mouse TFR1 act as an erythroid differentiation-responsive element[119]. Thus, translation of ALAS2 protein was increased along with a coordinate induction of TFR1 mRNA transcription under the condition replete with iron to synthetize enough

amount of heme for hemoglobin production during terminal differentiation of erythroid cells.

2. Heme-mediated translational regulation of ALAS-E

Ranu and London reported that rat reticulocyte lysates contained protein kinase, activity of which was regulated by heme, designated as heme regulated inhibitor (HRI) of translation[120]. HRI phosphorylates α subunit of eukaryotic initiation factor 2 (eIF2α), thereby inhibiting the translation of mRNAs[121]. HRI is a hemoprotein with two distinct types of heme binding site[122], and is autophosphorylated at multiple sites under heme deficient conditions[123, 124]. Without this autophosphorylation, HRI could not phosphorylate eIF2α, and unphosphorylated eIF2α is able to participate in the translation. Thus, increase of heme in erythroid cells during erythroid differentiation reduced a kinase activity of HRI, thereby inducing protein synthesis. Because globins are the predominantly synthesized proteins in mature erythroid progenitor cells, this system plays an important role to provide an appropriate amount of globin proteins for producing large amounts of hemoglobin during terminal differentiation of erythroid cells. Moreover, increased translational efficiency of ALAS2 mRNA should also account for synergistic production of hemoglobin synthesis in these cells[125].

C. Post-translational regulation of ALAS-E protein

1. Does heme inhibit translocation of ALAS-E into mitochondria?

As discussed above, ALAS-E is a nuclear encoded mitochondrial protein. Thus, ALAS-E protein is synthetised in cytosol as a precursor protein, then translocates into mitochondrial matrix, at where presequence is cleaved out to become mature protein. As demonstrated by Lathrop and Timko, N-terminal end of human ALAS2 precursor protein acts as a mitochondrial targeting sequence, and translocation of of precursor protein was inhibited by heme through heme reguratory motif (HRM) in vitro[50]. Cox et al. reportd that the region consisted of 49 amino acids at N-terminal end of human ALAS-E protein, which contains two HRMs (see Figure 3), is

sufficient for mitochondrial targeting and subsequent translocation into matrix[126]. However, it was still suspicious whether heme inhibit mitochondria translocation of ALAS-E in vivo, since ALAS-E expression is essential for hemoglobin production in eryhtroid cells, in which large amount of heme was produced[127].

Munakata et al. examined the relationship between intracellular heme and mitochondrial translocation of rat ALAS-E, and compared it with those of rat ALAS-N[51]. For this purpose, precursor protein of rat ALAS-N or ALAS-E was transiently expressed in Quail fibroblast cells, then the cells were treated with hemin or succinylaceton (SA), which is an effective inhibitor for heme biosynthesis, and their mitochondrial translocation was observed using Western blot analysis. As a result, authors found that mitochondrial translocation of expressed ALAS-N protein was severely impaired without any treatment, while the treatment of these cells with SA induced mitochondrial translocation of ALAS-N protein. Since expressed ALAS-N should be enzymatically active in transfected cells, intracellular heme should incleased, thereby inhibiting translocation of expressed ALAS-N. In contrast, transiently expressed rat ALAS-E could translocated into mitochondria without SA treatment, and addition of hemin into the culture medium did not give any noticeable effect. These results suggested that HRMs in rat ALAS-N are more sensitive to intracellular heme level than those in rat ALAS-E, and mitochondrial translocation of ALAS-E might be roughly regulated by heme compared to that of ALAS-N in vivo. However, it seemed to be reasonable that ALAS-E was hardly regulated by increased intracellular heme, since ALAS-E should be active in mitochondrial matrix to produce large amount of heme for hemoglobin formation in differentiating erythroid cells.

2. Post-translational regulation through interaction with the other proteins

After the translation as a precursor protein, ALAS-E protein should be associate with several proteins, such as chaperon proteins maintaining protein stability, translocation machinery at mitochondrial membrane and

processing peptidase that cleave out presequence in mitochondrial matrix. In this context, Furuyama and Sassa performed yeast two-hybrid assay using mature ALAS-E protein as a bait, and identified a possible association of ß subunit of ATP-specific succinyl CoA synthase (SCS-ßA) with human ALAS-E protein[128]. Succinyl CoA synthase (SCS) catalyzes phosphorylation of ADP or GDP, although SCS also catalyzes reverse reaction, in which condensate succinate and coenzyme A to produce succinyl CoA[129]. There are two isoforms of SCS, the ATP specific (A-SCS) and GTP-specific isoforms (G-SCS)[130]. It has been shown that these two isoforms of SCS contain the identical α subunit (SCS-α), although they differ in their ß subunits that determine the specificity of nucleotide binding[130, 131]. Furuyama and Sassa found that ß subunit of A-SCS (SCS-ßA) associate with ALAS-E, whereas ß subunit of G-SCS (SCS-ßG) or α subunit does not. Interaction between ALAS-N and any subunit of SCS was not observed; therefore the association of ALAS-E and A-SCS should be an erythroid-specific event. Since succinyl CoA is one of two substrates of ALAS-E, interaction of ALAS-E and SCS-ßA might useful to supply succinyl-CoA to ALAS-E efficiently. Moreover, SCS-ßA and several mutant ALAS-E proteins, which were identified in patients with X-linked sideroblastic anemia, were examined using yeast two-hybrid assay. As a result, two mutants of ALAS-E, which had been identified in patients with pyridoxine-responsive XLSA (Arg411Cys[132] and Met426Val[133]), showed similar interaction with SCS-ßA as did the wild-type ALAS-E, whereas a pyridoxine-refractory mutant (Asp190Val[133]) failed to interact with SCS-ßA. Because Asp190Val mutation resulted in an unstable enzyme in patient's bone marrow[133], it is possible that SCS-ßA play an important role for protecting ALAS-E from degradation in mitochondria[128].

Interaction between ALAS-E and SCS-ßA has been also confirmed by Cox et al[126]. These authors examined the strength of interaction between ALAS-E and SCS-ßA using yeast two-hybrid system, and found that it was relatively weaker than that of control proteins provided for determining weak interaction. The interaction between major splice valiant for ALAS-E (lacking 4thexon) and SCS-ßA was also examined, and it was found that they could interact in similar strength with that of wild type ALAS-E and SCS-ßA,

suggesting that amino acid region of ALAS-E protein encoded by exon 4 (102^{nd} -138^{th} amino acid of precursor human ALAS-E protein) was not involved in the interaction of ALAS-E and SCS-βA.

3. Processing of ALAS-E protein in mitochondria

Dzikaite et al reported the novel peptidase activity in bone marrow mitochondria, which cleave out N-terminal region of murine mature ALAS-E[134]. A series of experiments revealed that localization of this peptidase activity was restricted in mitochondrial membrane fraction isolated from bone marrow erythroblasts, and it was hardly detected or not detected in mitochondria isolated from peripheral blood erythrocyte or granulocytes, respectively. This peptidase produced approximate 7 kDa smaller ALAS-E protein than that expected as a mature murine ALAS-E protein (59 kDa), and it was distinct from mitochondrial proscessing peptidase (MPP), which cleaved the mitochondrial targeting signal in mitochondria. This newly identified peptidase was able to cleave murine ALAS-E precursor protein, as well as mature protein, in vitro, and its peptidase activity was inhibited by high dose α1-antitypsine in vitro, but not inhibited by EDTA, elastatinal, pepstatin, leupeptin, pefabloc, aprotinin or commercially available protease inhibitor cocktails. These authors demonstrated that smaller murine ALAS-E protein is localized at mitochondrial membrane fraction, and it should be active in vivo. It might be acceptable conclusion, however, biological significance of this peptidase activity and the production of the smaller ALAS-E protein in vivo is still obscure.

4. Hypoxia stabilizes ALAS-E protein

Using a data-base search, Abu-Farha et al. found that ALAS-E protein contained consensus sequence (L-X-X-L-A-P), in which proline residue is hydroxylated by proryl hydroxylase under normoxic condition (21% oxygen concentration), leading ubiquitination and subsequent degradation by proteasome[135]. Since this consensus sequence was commonly identified in human, rat, mouse, whale and zebrafish ALAS-E protein, but

not in ALAS-N protein, these authors examined the effect of hypoxia on the stabilization of ALAS-E protein. As a result, authors demonstrated that hypoxia or treatment with proteasome inhibitor induced ALAS activity in K562 erythroleukemia cells, and turn-over of the transiently expressed mature ALAS-E protein, which lacked its presequence, in K562 cells was prolonged by the treatment with hypoxia or proteasome inhibitor, as well as by an introduction of the mutation into hydroxylation consensus sequence of expressed ALAS-E protein. Moreover, authors confirmed that von Hippel-Lindau protein was co-immunoprecipitated with overexpressed mature ALAS-E protein is K562 cells, and mature ALAS-E protein was ubiquitinated under normoxic condition, but not under hypoxia, examined by in vitro ubiquitination system.

The concept of this research is interesting, and this kind of regulation could be occurred. However, FLAG-tagged ALAS-E protein examined in this paper remained in cytosol, since the expressed protein seemed to be lacked its mitochondrial targeting signal. Thus, it is still unclear whether precursor protein or intra-mitochondrial mature ALAS-E protein is ubiquitinated and degraded by proteasome.

VIII. The role of ALAS-E in hematopoiesis in vitro

A. Suppression of ALAS-E expression in murine cell line

Meguro et al. examined the effects of the stable expression of antisense mRNA for ALAS-E in murine erythroleukemia (MEL) cells[136]. Authors isolated several clones that expressed ALAS-E mRNA at various level, and found a strong positive relationship between ALAS-E mRNA level and ALA dehydatase, porphobillinogen deaminase, ferrochelatase, β-globim mRNA level or intracellular heme contents, either before or after induction of erythroid differentiation by dimethylsulfoxide (DMSO). Moreover, decrease of mRNA level for NF-E2 p45 protein was also correlated to the decrease of ALAS-E mRNA level in these cells. ALAS-N expression level in these cells was not different from that of control cells, suggesting that intracellular heme level was basically determined by the expression of

ALAS-E in MEL cells. Furthermore, decreased expression of these gene, except for ALAS-E mRNA, was partially recovered when the cells were treated with ALA or hemin, indicating that heme played an essential role for the expression of several genes related to erythroid differentiation in MEL cells.

B. In vitro differentiation of ALAS-E null ES cells

Harigae et al. examined the deletion effect of ALAS-E gene in mouse erythroblast, which were differentiated from ALAS-E targeted embryonic stem (ALAS-E(-)) cells. Authors induced erythroid differentiation of control ES cells and ALAS-E(-) cells in vitro, and found that mRNA for several transcription factors, including NF-E2 p45, EKLF and GATA-1, were similarly expressed in ALAS-E(-) cells derived erythroblast (ALAS-E(-) erythroblast), as well as in control ES cells derived erythroblasts. Moreover, Ter119-positive cells were also detected in ALAS-E(-) erythroblasts at similar frequency with those of control cells, suggesting that ALAS-E(-) erythroblasts could develop to a stage beyond colony forming unit-erythroid (CFU-E). The expression of β-major globin mRNA and protein in ALAS-E(-) erythroblasts were clearly decreased, and hemoglobin positive cells was not detected in ALAS-E(-) erythroblasts. However, decreases of β-major globin mRNA and protein level, as well as the disappearance of hemoglobin positive cells, were partially restored by the treatment of ALAS-E(-) erythroblasts with ALA during in vitro differentiation. Thus, these results suggested that the defect in heme synthesis in ALAS-E(-) erythroblasts was due to deficiency of ALA production by the lack of ALAS-E expression, and heme played a critical role in β-major globin synthesis and terminal differentiation of erythroblasts.

Yin and Dailey also reported a critical role of ALAS-E expression in heme production and subsequent hemoglobin formation during erythroblast differentiation from ES cells[137]. These authors pointed out that mRNA level of several genes involved in heme biosynthesis and embryonic β-globin gene were significantly decreased and absent in ALAS-E knockout ES cells, respectively, during early stage of the differentiation, suggesting that the

existence of interrelationship between heme and globin synthesis in differentiating ES cells to erythroblasts in vitro.

C. Heme-mediated regulation of the expression of other genes

In combination of in vitro differentiation of ALAS-E null ES cells and DNA array analysis, Fujiwara et al. tried to identify erythroid-specific heme-regulated genes[138]. For this purpose, authors performed differential expression analysis between wild-type and heme-deficient erythroblasts, which had been prepared from wild-type and ALAS-E null mouse ES cells, respectively. After an initial experiment, authors selected 120 genes, which expression was at least 3 times higher level in wild-type erythroblasts than that of ALAS-E null erythroblasts. Among of 120 initial candidate genes, 34 genes were expected to encode the protein with unknown function (referred as ESTs), while other 86 genes encoded previously characterized protein. Then, authors focused on 34 ESTs and 6 characterized genes, of which role in erythropoiesis was unclear. Based on their erythroid-lineage specific expression, high expression level in bone marrow and the positive relationship between intracellular heme level and their expression level, four genes were identified as erythroid-specific heme-regulated genes. These four gens were, uncoupling protein 2 (UCP2), nucleolar spindle-associated protein (NuSAP), cellular nucleic acid-binding protein (CNBP), and NST1, which encoded yet unknown protein. UCP2, NuSAP, and CNBP have been reported to be involved in the protection against reactive oxygen species[139], the regulation of cell cycles[140], and the regulation of cell growth[141], respectively. Authors speculated that a protection system, in which UCP2 is involved, might be essential to protect erythroblasts from certain cellular damage, since erythroblasts are exposed to reactive oxygen species generated by high concentration of iron during erythropoiesis. In addition, the cell cycle and growth of erythroblasts should be tightly regulated, in part by NuSAP and CNBP, respectively, to maintain the number of circulating red blood cells at appropriate level. Furthermore, authors performed database search to determine the putative function of EST1 gene, and found

that EST1 encoded the protein consists of 110 amino acids, and represented N-acetyltransferase domain, suggesting that this protein might belong to GNAT (GCN5-related N-acetyltransferase) superfamily. However, the specific function of these proteins in erythroid cells, and the mode of heme-mediated regulation of mRNA expression are still remained elusive.

IX. Essential role of ALAS-E in erythropoiesis and iron homeostasis in vivo

It has been reported that ALAS activity was decreased in erythroblasts or bone marrow of patients with sideroblastic anemia[142-144], and loss of function mutation of ALAS2 gene caused X-linked sideloblastic anemia[2, 93]. Thus several researchers have tried to establish the experimental model of sideroblastic anemia by decreasing the expression of ALAS-E in cultured vertebrate cells. However, the presence of ring sideroblasts, which is a hallmark for sideroblastic anemia, has never confirmed until the establishment of ALAS-E targeting mouse strain presented by Nakajima et al[145].

A. Role of ALAS-E expression in zebrafish

Brownlie et al. found that zebrafish mutant sauternes (sau) presented microcytic/hypochromic anemia, and positional cloning revealed that sau mutant carried missense mutation on ALAS-E gene[146]. Analyses of sau embryos revealed that hemoglobin production and number of erythroblasts in hematopoietic organs of sau embryos were significantly decreased than those of normal embryos. It was also found that the expression of ße2-globin mRNA and gata-1 mRNA was prolonged to the later stage of embryogenesis in sau mutant, whereas expression of these genes were clearly decreased at similar stage in normal embryos. Moreover, authors found that sau mutants could be raised to adulthood, and they showed a 2-3 folds increase in number of erythroblast in hematopoietic organ, and 5 folds increase of immature erythroid cells in circulation compared with wild type, indicating that similar phenotypes with those

observed in patient with X-linked sideroblastic anemia in human. Despite of several common phenotypic features between human congenital sideroblastic anemia and zebrafish sau mutants, ring sideroblasts, which is characterized by the iron deposition in erythroid mitochondria by iron staining of patient's bone marrow, was never been observed in hematopoietic organ in sau mutants. This critical difference in the formation of ring sideroblasts may be due to the differences in the mechanisms regulating iron homeostasis between zebrafish and mammals.

B. Targeting disruption of ALAS-E gene in mice

Nakajima et al. successfully established the female mice which stably maintaining disrupted ALAS-E gene mapped on X chromosome, and examined the effects of ALAS-E gene deletion in hemizygous male pups[145]. As a result, authors found that ALAS-E null embryo could not be viable after embryonic day 11.0 (E11.0), and blood vessel of E10.5 yolk sac of hemizygous embryo did not contain any hemoglobinized cells. The εy-globin protein expression in each blood cell of E10.5 was detected at similar level in wild-type and hemizygous embryo, while the number of such cells were significantly decreased in yolk sac of hemizygous embryo, indicating that absence of ALAS-E caused maturation arrest of erythroid cells and severely impaired primitive erythropoiesis. Further analyses revealed that primitive erythropoiesis of these cells was arrested at the stage after GATA-1 expression was initiated, and severe iron accumulation was observed in these cells, which was not detected in erythroid cells obtained from wild type embryo. Interestingly, accumulated iron was distributed in cytoplasm of erythroid cells isolated from ALAS-E null embryo, and it was quite different from that observed in ring sideroblasts, in which iron deposition was exclusively localized in mitochondria. Authors hypothesized that regulation of iron metabolism might be different between erythroblasts in primitive hematopoiesis and those in adult hematopoiesis, and searched the presence of ring sideroblasts in heterozygous male adult mice. As authors expected, typical ring sideroblasts were observed in bone marrow and peripheral blood corrected from adult chimeric female mice, even though the number of these

cells were limited. It should be notable that transferrin receptor 1 (TFR1) was abundantly expressed on the red blood cells of E8.5 ALAS-E null embryo, which was similar level with those of E8.5 wild type embryo. However, such abundant expression of TFR1 on circulating red blood cells was no more detected at E9.5 and 10.5, although abnormal iron accumulation in these circulating red blood cells was observed at E9.5 and it was further increased at E10.5, suggesting that hitherto unknown iron transport system, which might be independent from transferrin-TFR1 system, was involved in the abnormal cytosolic iron accumulation in hematopoietic cells of ALAS-E null embryo at E10.5. These results suggested that ablation of ALAS-E gene caused embryonic lethal phenotype because of severe anemia, and ALAS-E dependent heme biosynthesis was essential for hemoglobin production in primitive hematopoiesis. Moreover, targeted disruption of ALAS-E gene might alter the mechanism for maintaining iron homeostasis, and therefore revealed the difference in a mode of iron regulation in erythroid cells between primitive hematopoiesis in embryo and adult hematopoiesis.

Since ALAS-E null mice presented lethal phenotype at E11.0, the effect of ALAS-E deletion on definitive erythropoiesis could not be examined in vivo. Thus, Harigae et al. performed in vitro analysis, in which ES cells were differentiated to β-major globin positive definitive erythroblasts by co-culturing them on OP-9 stromal cells[147]. On the day 14, collected cultured erythroblasts differentiated from wild type (WT definitive erythroblasts) or ALAS-E null ES cells (ALAS-E null definitive eythroblasts) expressed adult β-major globin mRNA, whereas the expression of εy-embryonic globin mRNA could not be detected, suggesting that these cells were similarly differentiated to the level of definitive erythropoiesis. Expression level of several erythroid specific genes, such as GATA-1, NF-E2 p45 and DMT-1, in ALAS-E null definitive erythroblasts were also detected at similar level with those of WT definitive erythroblasts. Moreover, similar expression level of Ter119 was detected in the definitive erythroblasts derived from wild type and ALAS-E null ES cells. However, heme contents in ALAS-E null definitive erythroblasts were significantly decreased in ALAS-E null

definitive erythroblasts, and these erythroblasts were entirely colorless because of hemoglobin deficiency. These results suggested that ALAS-E expression was essential for hemoglobinization in definitive erythropoiesis, but lack of ALAS-E expression does not interfere the development to definitive erythropoiesis in vitro, although it causes a maturation arrest in primitive erythropoiesis in vivo. Furthermore, authors found that cytosolic non-heme iron accumulation, which was similar pattern observed in ALAS-E null mice embryos, was exclusively detected in ALAS-E null definitive erythroblasts, and such cells were more capable of oxidation of a fluorogenic substrate than were the WT definitive erythroblasts, suggesting that cytosolic iron accumulation in ALAS-E null definitive erythroblasts might increase the oxidative stress in these cells.

C. Transgenic rescue of ALAS-E null mice

Ring sideroblasts were not detected in primitive erythroblast isolated from ALAS-E null embryos, and further in vivo examination could not be performed because of embryonic lethal phenotype of ALAS-E null embryos[145]. Nakajima et al. thus tried to rescue of ALAS-E null mice by transgenic expression of wild type human ALAS-E cDNA for investigating developmental stage-specific pathogenesis of heme depletion[148]. First, authors established ALAS-E transgenic mice, in which human ALAS-E (referred as AE) was expressed at various levels under the control of mouse Gata-1 gene hematopoietic domain[149, 150]. Then authors selected three independent transgenic AE expressing lines of mouse, in which AE expressed at almost equal level with endogenous mouse ALAS-E expression (AE-H), at 50% less (AE-M) or 70% less (AE-L) than endogenous mouse ALAS-E during primitive and definitive erythropoiesis. Complementation rescue of AE-null mice using these lines of transgenic mice revealed that AE-H line could rescue embryonic lethal phenotype of ALAS-E null mice, while AE-L line could not show any noticeable effects on the correction of lethal phenotype at E11.5 of ALAS-E null mice. However, transgene of AE-M resulted in remarkable fluctuation in the efficiency of rescue of ALAS-E null mice. For example, one embryo found to be dead at around

E16.5, while another embryo from same litter was alive at E17.5, indicating that AE-M line could partially rescue the abnormal phenotype of ALAS-E null mice. Further analysis of partially rescued mice by AE-M transgene (designated AE-MR) revealed that the numerous number of typical ring sideroblasts were detected in the bone marrow at E12.5 by conventional Prussian blue staining, and the iron deposit in these cells were also confirmed by electron microscopy. Of quite interest, diffuse cytoplasmic iron accumulation in primitive erythroblasts, which was observed in ALAS-E null mice at E10.5, were also detected in AE-MR embryo at E11.5, suggesting that the iron-overloaded cells are most likely to be transformed into ring sideroblasts after E11.5. Moreover, typical ring sideroblasts were not detected in rescued embryo by AE-H transgene, suggesting that the iron overload in the erythroid cells of partially rescued embryos was provoked by the insufficient expression of ALAS-E from the transgene or heme-deficiency in the mutant erythroid cells. Prussian blue staining of peripheral blood cells (PBCs) from AE-MR embryo at E17.5 demonstrated the presence of ring sideroblasts-like nucleated erythrocyte, as well as a lot of enucleated red blood cells with a number of iron deposits, confirming the ring sideroblasts formation during definitive erythropoiesis.

These authors also examined the expression level of TFR1 on the surface of ring sideroblasts isolated from E12.5 AE-MR embryo, and found that TFR1 was expressed highly or moderately, which was not so different from that of wild type erythroblasts, suggesting that TFR1 might play important roles in the formation of ring sideroblasts in rescued embryo. Moreover, the results of FACS analyses detecting TFR1, Ter119 and c-kit on the surface of mononucleated cells isolated from E15 fetal liver suggested that the maturation arrest might be occurred in fetal liver of rescued mice.

Using transgenic rescue of ALAS-E null mice, these authors demonstrated first time that decreased expression of ALAS-E itself caused ring sideroblasts formation in mammalian hematopoietic organs. It was also important finding that the mechanisms for iron utilization in erythroblasts were dramatically changed during embryonic erythropoiesis, although the mechanisms for such regulation still remained unclear.

X. Summary and perspectives for the future

In this chapter, historical aspects related to the identification of ALAS and its' isozymes in vertebrates were described, and the different regulatory mechanisms for ALAS-N (ALAS1) and ALAS-E (ALAS2) were discussed. Expression of each isozyme was regulated quite differently, however, most important and critical difference should be the mode of heme-mediated regulation. It was demonstrated that ALAS-N expression was suppressed at transcriptional, translational and post-transcriptional level by increased free heme pool. This negative feedback mechanism plays a crucial role for maintaining heme homeostasis in non-erythroid cells, especially in hepatic cells. Since the expression of physiologically active heme transporter was not yet confirmed (at least by the end of 2011[151]), catabolism of heme regulated by heme oxygenases and suppression of heme biosynthesis, both of which were co-ordinary regulated by the fluctuation of free heme pool, may play a critical role for maintaining free heme pool at appropriate level within narrow range.

On the other hand, ALAS-E expression seemed to be almost free from the negative feedback regulation by heme in erythroid cells. It has been suggested that ALAS-E expression in erythroid cells was regulated by intracellular iron level rather than free heme pool[152]. In erythroblasts, large amount of heme should be produced for hemoglobin formation, for which synthesis of globin protein must be cooperatively induced during the terminal differentiation of erythroblasts. Increased heme enhances globin transcription by dissociating transcriptional repressor Bach1 from globin enhancer region[153, 154], and increases of free heme pool also mediates autopohsphorylation of heme regulated inhibitor (HRI)[123], thereby enhances globin protein translation. Even though excess amount of heme is produced over the binding capacity of globin protein or other hemoproteins, such free heme could be exported to the outside of erythroblast by heme exporter FLVCR[86]. Thus, free heme pool in erythroblasts could be maintained