Inactivation of G-protein-coupled Receptor 48 (Gpr48/Lgr4) Impairs Definitive Erythropoiesis at Midgestation through Down-regulation of the ATF4 Signaling Pathway

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G-protein-coupled receptors (GPCRs), one of the most versatile groups of cell surface receptors, can recognize specific ligands from neural, hormonal, and paracrine organs and regulate cell growth, proliferation, and differentiation. Gpr48/LGR4 is a recently identified orphan GPCR with unknown functions. To reveal the functions of Gpr48 in vivo, we generated Gpr48−/− mice and found that Gpr48−/− fetuses displayed transient anemia during midgestation and abnormal definitive erythropoiesis. The dramatic decrease of definitive erythroid precursors (Ter119pos population) in Gpr48−/− fetal liver at E13.5 was confirmed by histological analysis and blood smear assays. Real-time PCR analyses showed that in Gpr48−/− mice both adult hemoglobin α and β chains were decreased while embryonic hemoglobin chains (ξ, BH1, and e) were increased, providing another evidence for the impairment of definitive erythropoiesis. Furthermore, proliferation was suppressed in Gpr48−/− fetal liver with decreased c-Myc and cyclin D1 expression, whereas apoptosis was unaffected. ATF4, a key transcription factor in erythropoiesis, was down-regulated in Gpr48−/− fetal livers during midgestation stage through the cAMP-PKA-CREB pathway, suggesting that Gpr48 regulated definitive erythropoiesis through ATF4-mediated definitive erythropoiesis.

Erythropoiesis occurs sequentially in distinct anatomical locations in two different phases during embryogenesis. The earlier phase is defined as primitive erythropoiesis, which, in the mouse, originates from the yolk sac at embryonic day 7.5 (E7.5), and the later phase is definitive erythropoiesis, which was carried out in the fetal liver during midgestation after E12.5. Non-nucleated adult-type red blood cells are first generated in the fetal liver, the primary organ for erythropoiesis during midgestation from E12 to E16 (1). Erythropoiesis then transfers to bone marrow and spleen in the adult (2, 3). However, the molecular mechanism of regulating erythropoiesis has not been completely delineated. Most of the studies were focused on the transcription factors to explore the mechanisms of erythropoiesis (4, 5). In recent years, several transcription factors were also identified to regulate definitive erythropoiesis in fetal liver during midgestation (6–11). One of the transcription factors is ATF4, which has been shown to regulate cell proliferation in response to a broad spectrum of cell stresses and can be either an activator or a repressor in response to different extracellular signals (12). ATF4−/− mice have been reported to cause defective definitive erythropoiesis, and severe anemia at midgestation (13). Although receptors, such as c-Kit and EPOR, have been well studied in erythropoiesis (14), little is known on the function of G-protein-coupled receptors (GPCRs) in erythropoiesis during development (14, 15).

The GPCR family represents the largest and most versatile group of cell surface receptors (16–18). GPCRs can recognize their ligands, a diverse array of extracellular signals, then transmit these signals to intracellular responses by the ligand-receptor interaction. For its versatile roles, the GPCR family is one of the most promising and attractive targets to develop pharmaceutical drugs for human diseases ranging from allergic rhinitis to pain, hypertension, and schizophrenia (16). The glycoprotein hormone receptors represent a subgroup of GPCRs that have a large N-terminal extracellular (ecto-) domain containing leucine-rich repeats, a versatile structural domain that is important for glycoprotein hormone ligands recognition (19–21). Based on the comparison of peptide hormones and glycoprotein hormone receptors, a sub-group of GPCRs, leucinerich repeat-containing GPCRs (LGRs), was identified (22).
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Many studies suggest that the expanding family of LGRs, including three known glycoprotein hormone receptors (LH, FSH, and TSH), the orphan receptors LGR4 (also termed Gpr48), LGR5, and LGR6 are homologous and conservative (22–24). The mammalian glycoprotein hormone receptors have diverse structural features and mainly couple through the cAMP-dependent pathway for signal transduction (25).

Gpr48 (LGR4) has a putative horseshoe-like structure composed of 17 leucine-rich repeats, which is proposed to be the ligand-binding site for this family of receptors (21, 26). The molecular structure and evolutionary features of Gpr48 have been well studied; however, the ligands and physiological functions remain unclear (19, 20, 25, 27, 28). Gpr48 is widely expressed in multiple organs at both embryonic and adult stage (19, 27, 29), which suggests that Gpr48 might play a vital role in development and adult physiological functions. Recent studies from our and other laboratories have indicated that Gpr48 plays an important role in renal, eye, and reproductive system development (30–33). However, little is known thus far about the function and molecular mechanism of Gpr48 in erythropoiesis. In this study, we demonstrated that Gpr48 is expressed in both embryonic and adult liver, and that the deletion of Gpr48 in mouse impairs definitive erythropoiesis at midgestation through down-regulation of c-Myc, cyclin D1, and ATF4 pathways.

EXPERIMENTAL PROCEDURES

Generation of Gpr48 Knockout Mice—Gpr48 gene trap ES clone (LST020) was obtained from Bay Genomics (34, 35). The Gpr48 ES clones were injected into C57BL/6 blastocysts and transferred to ICR females. Male chimeric mice were mated with C57BL/6 females, resulting in transmission of the inserted allele to the germ line. Positive mice were interbred and main-

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Generation of Constitutive Activated Gpr48—PCR-based point mutagenesis was performed to generate mutant Gpr48 using cDNA encoding human Gpr48 receptors. To introduce the amino acid mutation at T755I into the wild-type cDNA of human Gpr48 gene, overlapping primers containing mutated sequences (forward: 5′-CGC TTG GCT AAT CTT CAT CAA TTG GGT G-3′; reverse: 5′-GCA ATT GAT GAA GAT TAG CCA G-3′) were used to replace the wild-type amino acid in the fetus and their wild-type littermates were harvested by flushing the femoral medullary cavity with Dulbecco’s modified Eagle’s medium. Cells are then centrifuged at 4°C 1500 rpm for 5 min followed by resuspending in phosphate-buffered saline (0.1 M and pH 7.2) and staining with anti-Ter119 (fluorescein isothiocyanate-conjugated) as a marker of mature erythrocyte for flow cytometry assay.

Histology and Immunohistochemistry—Embryos dissected from mice were fixed in 10% formalin and embedded in paraffin according to the standard techniques. 5-μm-thick sections were stained with hematoxylin and eosin. For proliferation assays, the sections were stained with anti-PCNA using the wedge technique followed by air drying and Wright-Giemsa staining (Sigma-Aldrich) following the manufacturer’s staining protocol. The number of nucleated erythrocytes and enucleated erythrocytes were counted in high power views.

Flow Cytometry—Single-cell suspensions were obtained from E13.5 wild-type and homozygous fetal livers. Briefly, fetal livers were dissected from embryos, then placed in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT) with 5% fetal bovine serum. The cells were dissociated by repeated flushing with a 23-gauge needle to obtain the single cell suspension, and the cell number was counted. Cell suspensions with the same concentration were first incubated on ice with rat anti-mouse CD16/CD32 (Pharmingen) to block nonspecific binding to Fc-receptors. Subsequently, cells were incubated with rat anti-mouse phycocerythrin-conjugated anti-c–Kit and anti-CD44, fluorescein isothiocyanate-conjugated anti-CD34 and anti-Ter-119 (all from Pharmingen). Appropriate isotype control antibodies were used. Then the cells were washed and transferred to phosphate-buffered saline 1% paraformaldehyde A. Cell surface expression of different markers was analyzed in a BD Biosciences FACScan using CellQuest software. To measure cell size of erythrocyte in fetal livers, liver cells from E13.5 Gpr48+/− fetus and their wild-type littermates were harvested by flushing the femoral medullary cavity with Dulbecco’s modified Eagle’s medium. Cells are then centrifuged at 4°C 1500 rpm for 5 min followed by resuspending in phosphate-buffered saline (0.1 M and pH 7.2) and staining with anti-Ter119 (fluorescein isothiocyanate-conjugated) as a marker of mature erythrocyte for flow cytometry assay.

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performed with 0.25 μg of plasmid using the Lipofectamine method following replacement of culture media. After 6–12 h of incubation with the Lipofectamine, media were replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. 48 h after transfection, cells were washed twice with Dulbecco’s phosphate-buffered saline, harvested from the plate. For luciferase transfection experiments, 48 h after transfection, cell lysate was collected, and luminescence assays were performed. Within a given assay, plate wells were set up in triplicate for each transfected construct or control vector. To allow for normalization of firefly luciferase values based on transfection efficiency, a co-reporter vector expressing β-galactosidase (pcDNA3-LacZ) was included at a ratio of 1:20 of co-reporter plasmid to experimental promoter construct (or control vector) in the transfection mixture.

Statistical Analysis—Data are presented as mean ± S.D. and analyzed by one-way analysis of variance or Student t test. For all analyses, p < 0.05 was considered statistically significant.

RESULTS

Targeted Inactivation of Gpr48 Gene and the Expression of Gpr48 in Fetal Liver—The murine Gpr48/LGR4 gene was disrupted by inserting a large secretory trap vector (11.98 kb) to
The Gpr48 ES clones were injected into C57BL/6 blastocysts to generate chimeric mice. Heterozygous Gpr48 mutant offspring were obtained by intercross between male chimera mice and C57BL/6 female mice. Heterozygous mice were intercrossed to obtain homozygous mice. The genotypes of offspring of mutant mice were confirmed by PCR analysis (supplemental Fig. S1B). The mRNA level of Gpr48 in the liver of homozygous, heterozygous, and wild-type mice was detected by RT-PCR analysis. The results showed that Gpr48 is expressed in the fetal liver of wild-type fetus, and that there was no expression of Gpr48 in homozygous mutant (Fig. 1A), confirming the successful deletion of Gpr48 in homozygous mutant. Because the mutant Gpr48 gene generates a chimeric protein containing the N-terminal leucine-rich repeat (LRRNT) domain and β-galactosidase, the expression patterns of Gpr48 in both heterozygous and homozygous mutant mice can be examined with the activity of β-galactosidase (LacZ staining) in fetal liver. As shown in Fig. 1B, LacZ staining was performed to measure β-galactosidase activity and the expression of Gpr48 in E15.5 fetal liver tissue sections. The expression of Gpr48 was found not only in hepatocytes but also in erythroid precursor cells (Fig. 1C). During midgestation, the erythroid precursors are mainly derived from liver cells, homologous to the hepatocytes. The expression of Gpr48 in erythroid precursor cells suggests a potential role of the receptor in erythropoiesis.

Deletion of Gpr48 Impairs Fetal Liver Size and Definitive Erythropoiesis—To understand the in vivo function of Gpr48 in fetal liver growth and erythropoiesis, we examined fetal liver weight and body weight in Gpr48−/− mice compared with wt mice. As shown in Fig. 2A, at E14.5, inactivation of Gpr48 gene affected liver growth with a 41% weight reduction (Fig. 2A, panel a) and 25% reduction in body weight (data not shown). Furthermore, the ratio of liver to body weight showed significant decrease (Fig. 2A, panel b), and the fetal livers of Gpr48 null embryos showed marked smaller size, indicating hypoplastic...
Development during fetal liver development from E12.5 to E16.5 (Fig. 2B). Because fetal liver is the predominant site for definitive erythropoiesis during embryonic stages, we further examined the morphological features of fetal liver from E12.5 to E16.5. As shown in Fig. 2B, Gpr48−/− embryos and fetal livers from E12.5 to E15.5 appeared considerably paler than their control wt littermates, suggesting less number of erythrocytes with low hemoglobin. However, little difference was found at E16.5 between wt and Gpr48−/− mice (Fig. 2B). In fetal liver, erythropoietic precursor cells can be distinguished from hepatocytes based on their smaller cell size and their more condensed and deeply stained nucleus. Histological examination and hematoxylin and eosin staining show that the gross morphologic features of homozygous fetal livers (green arrows) were normal in cellular architecture (Fig. 3). However, fewer erythroid precursor or definitive progenitors (yellow arrows) and erythroid foci (orange arrows) were visible in the homozygous fetal livers from E12.5–E15.5 embryos compared with that in wild type (Fig. 3). The fetal livers of Gpr48−/− embryos showed marked smaller size, indicating hypoplastic development during E12.5–E16.5.

Primitive erythrocytes are nucleated red blood cells in peripheral blood. From a blood smear, the nucleated erythrocytes showed the same size and shape in homozygous mice compared with that in wild-type, but significant less enucleated erythrocytes appeared in homozygous mice from E13.5 to E15.5 days (Fig. 4A). To quantify the nucleated erythrocytes, the number of both nucleated and enucleated erythrocyte was counted in the same high powder views. In Gpr48−/− embryos from E13.5 to E15.5, the percentage of nucleated erythrocytes was elevated 2.5-, 1.8-, and 1.5-fold compared with that in wild-type (Fig. 4B, p < 0.05, 0.01, and 0.01, respectively). However, no marked difference between homozygous and wild-type controls was found at E12.5 and E16.5 (p > 0.05). These nucleated erythrocytes in homozygous blood of these early embryos represented primitive erythrocytes produced by blood islands in yolk sac rather than the premature definitive erythrocyte releasing to blood stream from fetal liver.

The discrepancy of hemoglobin chain provides another way to identify these two kinds of erythrocytes. Primitive erythrocytes express embryonic globins (ζ, βH1, and ε) in contrast to nucleated erythrocytes in homozygous blood of these early embryos represented primitive erythrocytes produced by blood islands in yolk sac rather than the premature definitive erythrocytes releasing to blood stream from fetal liver.

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FIGURE 5. Flow cytometry analysis of fetal liver hematopoietic cells from E13.5 Gpr48+/+ and Gpr48−/− embryos. Cell suspensions from E13.5 fetal livers were labeled with anti-c-kit, anti-CD34, anti-CD44, or anti-Ter119-specific antibody. The populations of Ter119-positive cells, which represent definitive erythrocytes in Gpr48−/− embryos, were markedly decreased.
that definitive erythrocytes express adult forms of hemoglobins (β and α) (2, 38–40). Real-time PCR analyses were performed to examine the mRNA levels of different kinds of globins. In E13.5 embryos, the mRNA expression levels of embryonic globins ζ, βH1, and εγ from blood samples showed marked increase in Gpr48−/− fetal liver compared with wt livers as shown in Fig. 2B. This result was consistent with the elevated ratio of primitive erythrocytes in the blood of homozygous mice (Fig. 4, A and B). On the other hand, the fetal liver is the primary site to produce adult forms of hemoglobins, the expression levels of α and β globin mRNAs in the liver of Gpr48−/− mice at E13.5 showed significantly decreased (p < 0.01 and p < 0.05, respectively) (Fig. 4D), indicating that deletion of Gpr48 affected definitive erythropoiesis in fetal liver rather than primitive erythropoiesis. The decreased α and β globin expression also implies a smaller erythrocyte size in Gpr48−/− fetal livers. Indeed, the red blood cell size of the Ter-119-positive population, representing committed erythroid cells in definitive erythropoiesis (5, 10), was decreased in E13.5 Gpr48−/− fetal livers (Fig. 4E, x-axis represents cell size), indicating deletion of Gpr48 reduced red blood cell size by regulating the expression levels of mature hemoglobins. Furthermore, the less amounts of adult forms of globins lead to the paler appearance of the described above, both the number of liver cells and definitive erythroid precursors in Gpr48−/− fetal liver actually decreased. The reduction of definitive erythroid progenitors and liver cells in Gpr48−/− fetal liver might be associated with either decreased cell proliferation or increased apoptosis. Immuno-histochemical assays of PCNA and BrdUrd incorporation assays indicated that the number of proliferating cells was markedly reduced in the fetal liver of homozygous Gpr48−/− mice compared with their control littersmates (Fig. 6A for PCNA and Fig. 6B for BrdUrd labeling). However, no marked differences were observed in the apoptosis of fetal liver cells using TUNEL assays at E13.5 (Fig. 6C).

To understand the mechanism of how Gpr48 regulates cell proliferation, we examined the expression levels of key proteins involved in cell proliferation in Gpr48−/− fetal liver. As shown in Fig. 7A, both c-Myc and cyclin D1 were markedly reduced in Gpr48−/− mouse liver at E13.5 and E14.5 using Western blot assays (Fig. 7A). This result was confirmed by immunohistochemistry staining with specific antibodies for c-Myc and cyclin D1, respectively (Fig. 7, B and C). Both cyclin D1 and c-Myc play critical roles in cell proliferation and the cell cycle. Cyclin D1, expressed at the stage of G1, is the downstream gene of c-Myc. The proliferation of mammalian cells is governed by cyclins and their
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**A**

|       | +/+ | -/- | +/+ | -/- |
|-------|-----|-----|-----|-----|
| C-Myc|     |     |     |     |
| Cyclin D1|     |     |     |     |
| Actin |     |     |     |     |

E13.5  E14.5

**B**

Gpr48+/+  Gpr48-/−

c-Myc

**C**

Cyclin D1

FIGURE 7. Down-regulation of c-Myc and Cyclin D1 in Gpr48−/− fetal liver. A, Western blot analysis showed that protein levels of c-Myc and Cyclin D1 were both decreased in Gpr48−/− fetal livers compared with those in the wild type. Actin was used as an internal control. B, fetal liver sections from E13.5 Gpr48+/+ and Gpr48−/− embryos were stained with anti-c-Myc antibody. Significant decrease of c-Myc protein level was observed in Gpr48−/− fetal liver (brown color, magnification, 1000×). C, decreased protein levels of Cyclin D1 in Gpr48−/− fetal liver compared with wild-type. Fetal liver sections from E13.5 Gpr48+/+ and Gpr48−/− embryos were stained with anti-Cyclin D1 antibody (brown color, magnification, 1000×).

associated cyclin-dependent kinases (CDKs) (41). These cyclin-CDK complexes phosphorylate critical cellular substrates, thereby allowing the ordered progression of the cell cycle (42).

**ATF4** Is a Potential Target Gene of Gpr48 in Regulating Definitive Erythropoiesis—ATF4 has been shown to regulate cell proliferation in response to a broad spectrum of cell stresses and can be either an activator or a repressor in response to different extracellular signals (12). ATF4−/− affects the development of multiple organs or systems such as skeleton (43, 44), lens (45), liver (13), and reproductive system (46). ATF4−/− mice have defective definitive erythropoiesis and severe anemia at midgestation (13). Interestingly, all of those implicated tissues in ATF4−/− mice express high levels of Gpr48. Gpr48−/− and ATF4−/− mice have similar phenotypes (29–31). To determine whether Gpr48-mediated anemia at midgestation is caused by the regulation of ATF4 transcription factor, we measured the expression level of ATF4 using different approaches. As shown in Fig. 8, deletion of Gpr48 markedly down-regulated the mRNA expression levels of ATF4 in fetal livers from E13.5 to E15.5 days using RT-PCR and real-time PCR assays (Fig. 8, A and B). To determine whether the ATF4 protein was affected in Gpr48−/− mice, we performed Western blot and immunohistochemistry staining with specific anti-ATF4 antibodies. The protein expression level of ATF4 in Gpr48−/− fetal liver was significantly decreased by both Western blot (Fig. 8C) and by immunohistochemistry analysis (Fig. 8D). These results suggest that ATF4 is a downstream target of Gpr48, and the impairment of definitive erythropoiesis in Gpr48−/− fetal embryos at midgestation was likely caused by the marked reduction of ATF4 expression in Gpr48−/− fetal liver.

Gpr48 Regulates the Intracellular Activation through the cAMP-PKA-ATF4 Signaling Cascade—ATF4 (also termed CREB 2) is a member of CREB family of transcription factors, a major downstream transcription factor family of the cAMP-PKA pathway. Because the ligands of Gpr48 remain unclear at present, therefore generation of constitutively active mutant receptor provides a ligand-independent method for the function assay of Gpr48. Based on early studies found in FSH and LH receptors, we generated a number of single mutations using specific site-direct mutagenesis method, and we then examined the intracellular cAMP production and activation of cAMP-downstream transcription factor. One of the mutations, Gpr48 T755I, was found to increase intracellular cAMP significantly compared with the control and wild-type receptor without any ligand stimulation (Weng et al. (60)). The result was independently confirmed by Dr. Kitagawa’s group (47), suggesting that G-protein Gas and cAMP pathways are coupled to Gpr48. To further explore the molecular mechanism how Gpr48 regulates ATF4, we examined the proximal promoter region of ATF4 promoter and found a semi-CRE binding site CGTCA (at position −921 relative to the transcription start site) for potential binding and activation of the ATF4 gene by the CREB transcription factor and the cAMP-PKA signaling pathway. To confirm that Gpr48-mediated cAMP-CREB pathways regulate the promoter activity of ATF4, we cotransfected the constitutively active mutant Gpr48 (T755I) and a 1.2-kb ATF4 promoter-luciferase reporter gene into the cells, and then examined the direct...
activation of ATF4 by Gpr48 and cAMP pathways. As shown in Fig. 8E, the Gpr48-active mutant receptor (T755I) significantly increased the intracellular cAMP production. The activation of cAMP level by Gpr48 active receptor was significantly inhibited by adding PKI, a competitive PKA inhibitor (Fig. 8E). To further determine whether Gpr48 regulates ATF4 through PKA, we also used another PKA inhibitor, H89, a chemical PKA inhibitor, to measure ATF4 promoter activation mediated by the Gpr48-cAMP-PKA pathway. As shown in Fig. 8E, H89 successfully inhibited the activation of ATF4 promoter activated by Gpr48 active mutant receptor (T755I) (Fig. 8E). These data indicate that ATF4 was the direct downstream target gene of the Gpr48-mediated cAMP-PKA-CREB signaling pathway.

**DISCUSSION**

The present study demonstrated that inactivation of Gpr48 (LGR4) lead to impairment of definitive erythropoiesis at midgestation in fetal liver. Using a gene-trapped approach described previously (36), we constructed the Gpr48 inactivation model by inserting a secretary-trap vector (11.98 kb) at intron 1 of wild-type Gpr48. Ablation of Gpr48 resulted in the impairment of definitive erythropoiesis at midgestation, which is associated with the down-regulation in the expression levels of c-Myc, cyclin D1, and ATF4 through the cAMP-PKA-CREB signaling pathway. Gpr48 is expressed in a wide range of tissues, including skeleton, cartilage, ovary, testis, adrenal, thyroid, kidney, heart, and liver (19, 27, 29), suggesting that Gpr48 is involved in the developmental regulation of multiple organs, including fetal liver. The facts that Gpr48 is highly expressed in the fetal liver and in the membrane of premature erythroblast at midgestation indicate that Gpr48 play a key role for the development of definitive erythropoiesis. Erythropoiesis includes two sequential process termed primitive erythropoiesis and definitive erythropoiesis, respectively. Our data indicated that the silencing of Gpr48 had no evident effects on primitive erythropoiesis but dramatically reduced definitive erythropoiesis. Based on different characteristics between definitive erythropoiesis and primitive erythropoiesis (2, 16), we counted the nucleated erythrocytes (primitive erythrocyte) and enucleated erythrocytes (definitive eryth-
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erythrocytes) from blood smear and distinguish these two populations by real-time PCR arrays for different globin chains. The elevated level of nucleated erythrocytes indicated that more primitive erythrocytes in the circulation of Gpr48−/− mice, which was consistent with the result of real-time PCR analysis for globin chains (α, βH1, and ε). However, adult α and β globin chains was decreased markedly, indicating reduced definitive erythrocytes in Gpr48−/− mice. Examinations of the hemoglobin chain suggest that the relative increased number of nucleated erythrocytes in homozygous mutants resulted from persistence of primitive yolk sac-derived erythrocytes rather than the premature release of nucleated definitive erythrocytes into circulation due to stress erythropoiesis.

ATF4, also termed CREB2, is a member of the ATF/CREB ubiquitous basic leucine zipper (bZip) transcription factors. ATF4 is essential for cell proliferation (13, 43), especially for the processes that require high level proliferation, such as fetal liver hematopoiesis (13), bone development (45), lens development (44), and human cancer cells (48). ATF4−/− mice showed severe anemia due to impairment of definitive erythropoiesis during midgestation, indicating that ATF4 plays a pivotal role for definitive fetal-liver erythropoiesis under stress response (13). Similar to ATF4−/− mice, Gpr48−/− fetal livers also have elevated nucleated primitive erythrocytes relative to enucleated definitive erythrocytes without morphologically changes of primitive erythrocytes. Here we provide evidences that Gpr48 regulates the expression level of ATF4 through the cAMP-PKA-CREB signaling pathway. As a critical transcription factor in response to hypoxia, ATF4 activates its downstream gene expression, including CHOP and TRB3 (49, 50). These genes are stress-induced factors and collaborate to modulate a complex regulation network for fulfilling a fine regulation under conditions of integrated stress response or erythropoiesis (50–52). Furthermore, the pathway of signaling transduction for ATF4-CHOP-TRB3 might be independent of HIF-1α (49), a critical factor to mediate hematopoiesis through the inducing EPO pathway.

One of the most important changes in erythroid development is the ongoing maturation and the loss of proliferative capacity in favor of the differentiation program. The balance between erythropoietin (Epo)-mediated erythroid differentiation and stem cell factor-mediated proliferation are strictly regulated during erythropoiesis. In the presence of Epo, Signal transducer and activator of transcription 5 (STAT5) is recruited to the Epo receptor and phosphorylated by the Janus kinase 2 (JAK2). Once phosphorylated, STAT5 is released from EpoR, homodimerizes in the cytosol, and translocates to the nucleus where it activates its target gene expression, including Bcl-X and cyclin D1 (53–58). Phosphorylation of CREB by PKA enhances Epo-stimulated STAT5 transactivation by inducing recruitment of CREB/CBP/p300 to the STAT5 transactivation complex (58, 59). Inactivation of Gpr48 caused dramatic decrease in the proliferation of definitive erythroid progenitors and erythroblast islands in fetal liver. Moreover, analyses of key proliferation genes demonstrate that both c-Myc and cyclin D1 were markedly down-regulated in Gpr48−/− fetal liver. These data suggest that Gpr48 also affect definitive erythropoiesis by regulating Epo-mediated erythroid differentiation through the cAMP-PKA-CREB pathway.

The erythropoiesis of Gpr48−/− embryos was mainly affected during E12.5–E15.5 with E13.5 as the most severe stage. After E15.5, the anemia phenotype in a blood smear of Gpr48−/− mice looked gradually normal, suggesting that the defect of definitive erythropoiesis in Gpr48 homozygous mice is a transient process at midgestation. Other factors might serve as compensatory regulators for Gpr48−/− embryos surviving through midgestation. These compensatory genes might be expressed a bit later than ATF4 during the onset of fetal liver hematopoiesis to coordinate with ATF4 and to regulate hematopoiesis under Gpr48 ablation.

In summary, Gpr48 ablation resulted in impaired fetal-liver definitive erythropoiesis at midgestation. A reduced population of definitive erythrocytes was associated with decreased proliferation and down-regulation of c-Myc and cyclin D1 in Gpr48−/− fetal liver. These results suggest that Gpr48 is a critical GPCR to regulate fetal liver erythropoiesis during midgestation stage through intracellular signaling pathways coupled to the regulation of ATF4.

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