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Synergistic Role of Igf2 and Dlk1 in Fetal Liver Development and Hematopoiesis in Bi-Maternal Mice

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Abstract. Mouse bi-maternal embryos (BMEs) that contain two haploid sets of genomes from non-growing (ng) and fully-grown (fg) oocytes develop to embryonic day (E) 13.5. However, the ng/fg BMEs never develop beyond E13.5 because of repression of the paternally expressed imprinted genes, Igf2 and Dlk1. The present study was conducted to address the issue of whether fetal hematopoietic disorder is involved in the restricted development of BMEs. FACS analysis revealed that the livers of ngΔch7/fg BMEs contained increased numbers of immature c-kit+/ter119– hematopoietic cells, while the numbers of mature c-kit+/ter119+ hematopoietic cells were decreased. This finding was supported by histological observations. Quantitative gene expression analysis revealed that Igf2 and Dlk1 expression was repressed in the liver. To understand the role of paternally-methylated imprinted genes on expression, the proportions of mature and immature hematopoietic cells in the livers of the ngΔch7/fg wt/fg ΔDouble BMEs using ng oocytes harboring deletion of differentially methylated regions at distal chromosomes 7 and/or 12. The ngΔch7/fg, ngΔch12/fg and ngΔDouble/fg BMEs, respectively, express Igf2, Dlk1 and both, and these embryos developed to term with specific phenotypes; the ngΔch7/fg and ngΔch12/fg BMEs develop to term with severe growth retardation, and the ngΔDouble/fg BMEs can survive to become normal female adults. By inducing Igf2 and Dlk1 expression, the proportions of mature and immature hematopoietic cells in the livers of the ngΔch7/fg, ngΔch12/fg and ngΔDouble/fg BMEs were considerably restored, and particularly in the ngΔDouble/fg BMEs, hematopoiesis occurred normally with appropriate expressions of the related genes. These data suggest that inappropriate expression of Igf2 and Dlk1 is involved in impaired fetal hematopoiesis.

Key words: Dlk1, Genomic imprinting, Hematopoiesis, Igf2, Liver, Mouse

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sion analyses in the fetal livers of ng/fg bi-maternal embryos, and performed a FACS analysis of fetal liver cells to demonstrate the differentiation of erythroid cells using antibodies against ter119, a mouse erythroid-specific marker, and c-kit, a marker for immature hematopoietic cells. The results showed defection of fetal liver development and hematopoiesis in the ng/fg BMEs, suggesting a synergistic role for paternally methylated imprinted genes on chromosomes 7 and 12 in fetal liver development and hematopoiesis in the bi-maternal mouse fetuses. The defective liver development and hematopoiesis could be one of the causes of arrested development in the ng/fg BMEs.

**Materials and Methods**

**Nuclear transfer**

The ng wt/fg, ng Δch7/fg, ng Δch12/fg and ng ΔDouble/fg BMEs were reconstructed according to our previous reports [7]. In brief, fully grown germinal vesicle (GV) oocytes were collected into M2 medium from the ovarian follicles of B6D2F1 (C57BL/6N × DBA) female mice 44–48 h after they were injected with equine chorionic gonadotrophin. Ovulated MII oocytes were also collected from superovulated B6D2F1 mice 16 h after they were injected with human chronic gonadotrophin. We collected ng oocytes that were in the diplotene stage of first meiosis from the ovaries of 1-day-old newborn mice. The GV oocytes were manipulated in M2 medium containing 200 μM dbcAMP and 5% calf serum throughout the experiment. The ng oocytes were fused with enucleated GV oocytes by using inactivated Sendai virus (HVJ, 2700 hemagglutinating activity unit/ml). The reconstructed oocytes were also collected from superovulated B6D2F1 mice 16 h after they were injected with human chronic gonadotrophin. We collected ng oocytes that were in the diplotene stage of first meiosis from the ovaries of 1-day-old newborn mice. The GV oocytes were manipulated in M2 medium containing 200 μM dbcAMP and 5% calf serum throughout the experiment. The ng oocytes were fused with enucleated GV oocytes by using inactivated Sendai virus (HVJ, 2700 hemagglutinating activity unit/ml). The reconstructed oocytes were also cultured in ovulated MII oocytes, followed by treatment with 10 mM SrCl2 in Ca2+-free M16 medium for 2 h. These embryos were cultured in M16 medium for 3.5 days in an atmosphere of 5% CO2, 5% O2 and 90% N2 at 37°C. The embryos that developed to the blastocyst stage were transferred into the uterine horns of recipient female mice at 2.5 days of pseudopregnancy. All the mice were maintained and used in accordance with the guidelines for the care and use of laboratory animals specified by the Japanese Association for Laboratory Animal Science and by the Tokyo University of Agriculture.

**Histological analysis**

For histological analysis, the livers of mouse BMEs (ng wt/fg, ng Δch7/fg, ng Δch12/fg and ng ΔDouble/fg) and control fetuses (wt) at day 12.5 of gestation were fixed in 4% freshly prepared paraformaldehyde (Sigma-Aldrich Chemical, St. Louis, MO, USA) in PBS at 4°C. Next, formalin-fixed paraffin-embedded sections of the livers were stained with hematoxylin and eosin (HE) using standard techniques.

**In situ hybridization (ISH)**

At day 12.5 of gestation, the livers of the BMEs (ng wt/fg, ng Δch7/fg, ng Δch12/fg and ng ΔDouble/fg) and control fetuses (wt) were fixed in 4% freshly prepared paraformaldehyde in PBS. The livers were incubated in 10–20% graded sucrose and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). Sections (10 μm) of the liver tissues were placed on the slides, and ISH was performed essentially as described previously. Digoxigenin-labeled RNA probes for ISH were prepared using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. To obtain the RNA probes, cDNA fragments of Igf2 (primers: 5'-GAC TCT GTG CGG AGG GGA GC-3', and 5'-TCT TTG GGT GGT AAC ACG AT-3') and Dlk1 (primers: 5'-CCT CTT GCT CCT GCT GGC TTT C-3' and 5'-GAT GTG TTG CTC GGG CTG CTG A-3') were amplified by reverse transcriptase (RT)-PCR and inserted in a pGEM®-T Easy Vector (Promega, Madison, WI, USA).

**Quantitative analysis of gene expression**

At days 12.5, 15.5 and 18.5 of gestation, total RNA was extracted from the livers of the 4 genotypes of BMEs (ng wt/fg, ng Δch7/fg, ng Δch12/fg and ng ΔDouble/fg) and the control (wt) using ISOGEN (Nippon Gene, Tokyo, Japan). The cDNAs were synthe-
sized using a SuperScript™III RNase H- Reverse Transcriptase kit (Invitrogen™) in a reaction solution (20 μl) containing total RNA (1 μg) prepared from the tissues. Subsequently, the cDNAs were used for quantitative analysis of genes expression. This analysis was performed using real-time quantitative PCR (LightCycler™ System; Roche Diagnostics) with a ready-to-use reaction mixture kit (LightCycler FirstStart DNA Master SYBR Green I; Roche Diagnostics). The sequences of the primers used for the PCR reaction and the corresponding GenBank accession numbers are listed in Table 1. The GAPDH gene was used as a loading control.

Flow cytometry

At E12.5, the livers of the BMEs (ngΔch7/fg, ngΔch12/fg and ngΔch12/Δch12/fg) and controls (wt) were mechanically dissociated by pushing them with a syringe plunger through a 40-mm strainer in the presence of phosphate-buffered saline (PBS) and 0.5% bovine serum albumin (PBS/0.5% BSA). Single-cell suspensions (5 x 10^5) were immunostained for 1 h at 4°C in PBS/0.5% BSA with fluorescein isothiocyanate (FITC)-conjugated anti-c-Kit (1 mg/ml) antibodies and phycoerythrin (PE)-conjugated anti-Ter119 (1 mg/ml) antibodies (Pharmingen, San Diego, CA, USA). After 2 washes, flow cytometry was conducted on a Becton Dickinson FACSCalibur (Franklin Lakes, NJ, USA), and 10,000 events were acquired for each sample.

Statistical analysis

The difference in the levels of gene expression between the BME and control were determined by ANOVA and a t-test (a P value of less than 0.01 was considered statistically significant).

Results

Histological examination of the fetal livers

In E12.5 wt fetal livers, obvious intermingling of well-differentiated hematopoietic precursors and hepatic cells was observed in the parenchyma (Fig. 1A); however, differentiation was apparently abnormal in the ngΔch7/fg BMEs. The number of parenchymal cells and hematopoietic precursors were decreased considerably in the unit area with increasing frequency of aggregated and/or fragmented nuclei in the liver of the ngΔch7/fg BMEs (Fig. 1B). These prominent disorders were obviously restored in the livers of the ngΔch7/fg, ngΔch12/fg and ngΔdouble/fg BMEs, which were morphologically similar to that of the wt liver (Figs. 1C, D and E). These results suggest that the differential disorders in the fetal livers might be involved in restricted development of ngΔch7/fg BMEs to E13.5.

Expression of Igf2 and Dlk1 in fetal livers

To understand the liver-specific expression of the imprinted Igf2 and Dlk1 genes, quantitative gene expression and ISH analyses were performed at E12.5 (Figs. 2A). With regard to the ngΔch7/fg and ngΔch12/fg BMEs, the Igf2 and Dlk1 genes were inversely expressed and repressed and were 75% and 20% and 0.4% and 93% of those in the wt livers, respectively. The expression levels of both genes in the ngΔdouble/fg BME livers were expressed at the same levels as those in the wt livers. In the ngΔch7/fg BME livers, expression of Igf2 and Dlk1 was extremely low. To show tissue-specific expression, ISH analysis was carried out (Fig. 2B). Distinct signals for Igf2 and Dlk1 in the parenchyma of the liver anlage were detected in the ngΔch7/fg and ngΔch12/fg livers, respectively. In the ngΔdouble/fg BME livers, both signals for Igf2 and Dlk1 were detected clearly in parenchyma of the liver anlage.

Definitive erythropoiesis in BMEs livers

In order to obtain further insights into fetal hepatopoesis, we performed a FACS analysis of the fetal liver cells to demonstrate the differentiation of erythroid cells using antibodies against ter119, a mouse erythroid-specific marker, and c-kit, a marker for immature hematopoietic cells (Fig. 3A and Table 2). Of the fetal liver cells obtained from wt embryos, >67% and 15% were c-kit+/Ter119+ and c-kit+/ter119-, respectively; this suggests that a majority of the cells differentiated into erythroblasts (Fig. 3A). In contrast, the differentiation of erythroblasts was limited in the ngΔch7/fg BMEs; 42% of the cells were c-kit+/Ter119+ and 35% were c-kit-/Ter119-. Furthermore, the proportion of c-kit+/Ter119+ cells was significantly higher in the ngΔch7/fg BMEs compared with that in the wt cells, i.e., 16% vs. 10%. This disorder in erythroid differentiation was significantly restored in the ngΔch7/fg, ngΔch12/fg and ngΔdouble/fg BMEs, with a significant increase in the number of c-kit+/Ter119+ cells (62, 61 and 65%, respectively) and a significant decrease in the number of c-kit-/Ter119- cells (16, 24 and 15%, respectively; Fig. 3A, ngΔch7/fg and ngΔch12/fg). The proportion of c-kit+/Ter119+ cells did not change significantly, and this suggested that differentiation from c-kit+/Ter119+ cells to c-kit-/Ter119- cells was accelerated in the ngΔch7/fg, ngΔch12/fg and ngΔdouble/fg BMEs livers.

Expression of erythropoiesis related genes

We also investigated the expression of erythroid hematopoietic marker genes in the fetal livers at E12.5 (Fig. 3B). Expression of the Gata1, EpoR, Eklf, and NF-2E genes was evidently decreased in the ngΔch7/fg and ngΔch7/fg BME fetal livers. The expression levels were less than half those observed in the wt fetal livers with the exception of Eklf expression. Although the ngΔch7/fg BMEs developed to E19.5, the expression levels of these genes did not recover. The expression levels of these genes were elevated in the ngΔdouble/fg BME livers but did not recover to the levels in the wt livers. The expression of the erythroid hematopoietic markers in the ngΔdouble/fg BME livers was increased to the levels somewhat similar to those in the wt liver (Fig. 3B). Together with the FACS data, these observations suggest that the inappropriate expression of Igf2 and Dlk1 is a major cause of definitive erythropoiesis in the ngΔch7/fg BME fetal livers.

Discussion

In the present study, we investigated the development of the embryonic hematopoietic system in BME livers because the hematopoietic system is critical for survival of embryos beyond mid-gestation. Bi-maternal mouse embryos in which the paternally-methylated and paternally-expressed imprinted genes containing Igf2 and Dlk1 are fundamentally repressed resulted in lethality before E13.5, accompanied by acute hematopoietic disor-
Experiments involving introduction of \( \text{Igf2} \) and/or \( \text{Dlk1} \) expression in mutant BMEs early demonstrated significant amelioration of fetal liver hematopoiesis. The fetal liver functions as a major site for hematopoiesis at mid-gestation. To date, several studies have reported that the imprinted \( \text{Igf2} \) and \( \text{Dlk1} \) genes are involved in the development of fetal liver hematopoiesis.

During the first half of the embryonic period, the space between the hepatic cell cords expands, allowing for the development of hematopoietic precursors and the formation of intrahepatic microvessels. These structures provide a favorable environment for the proliferation and differentiation of hematopoietic cells. The expression of \( \text{Igf2} \) and \( \text{Dlk1} \) genes in the liver at E12.5 was quantitatively analyzed by real-time PCR. The expression of each gene is shown as the average of the expression levels relative to those of the \( \text{Actb} \) gene, which was used as an internal control. Data are expressed as means ± SEM (n=5). Stars indicate significant differences from the controls (P<0.01).

Definitive erythropoiesis in the fetal liver of the ng\(^{wt/fg}\), ng\(^{Δch7/fg}\), ng\(^{Δch12/fg}\) and ng\(^{ΔDouble/fg}\) BMEs at E12.5 was analyzed using FACS analysis and real-time PCR. The expression of \( \text{GATA1, EpoR, EKLF and NF-2E} \) expression were quantitatively analyzed by real-time PCR. The expression of each gene is shown as the average of the expression levels relative to those of the \( \text{Actb} \) gene. Data are expressed as means ± SEM (n=5). Asterisks indicate significant differences from the controls (P<0.01).

Histological examination of the fetal livers. Sections of the fetal livers obtained from (wt) mice (A) and the 4 BMEs, ng\(^{wt/fg}\) (B), ng\(^{Δch7/fg}\) (C), ng\(^{Δch12/fg}\) (D) and ng\(^{ΔDouble/fg}\) (E), at E12.5 stained with hematoxylin and eosin (HE). Developing parenchymal hepatocytes (PH), hematopoietic precursors (HP), intrahepatic microvessels (IM) and megakaryoblasts (M) were visible in the liver. Reduction of the cytoplasm (black arrow), condensation of the nucleus (white arrow) and fragmentation of the nucleus (arrowhead) were observed in the liver parenchyma of the ng\(^{wt/fg}\) BME (arrow). Scale bar=10-mm.

Fig. 2. Expression of the \( \text{Igf2} \) and \( \text{Dlk1} \) genes in the liver at E12.5. (A) \( \text{Igf2} \) and \( \text{Dlk1} \) expression were quantitatively analyzed by real-time PCR. The expression of each gene is shown as the average of the expression levels relative to those of the \( \text{Actb} \) gene, which was used as an internal control. Data are expressed as means ± SEM (n=5). Stars indicate significant differences from the controls (P<0.01). (B) \( \text{Igf2} \) and \( \text{Dlk1} \) expression were assessed in sections of the BMEs livers at E12.5 using in situ hybridization, and dioxigenin-labeled RNA probes were prepared using a DIG RNA labeling Kit (Roche Diagnostics GmbH, Mannheim, Germany). Scale bar=20-mm.

Fig. 3. Definitive erythropoiesis in the fetal liver of the ng\(^{wt/fg}\), ng\(^{Δch7/fg}\), ng\(^{Δch12/fg}\) and ng\(^{ΔDouble/fg}\) BMEs at E12.5. (A) FACS analysis of the 3 BME livers cells at E12.5. Single fetal liver cells obtained from a wt mouse and BMEs were immunostained with anti-Ter119 and anti-c-Kit antibodies. Liver cells were analyzed by flow cytometry. (B) \( \text{GATA1, EpoR, EKLF and NF-2E} \) expression were quantitatively analyzed by real-time PCR. The expression of each gene is shown as the average of the expression levels relative to those of the \( \text{Actb} \) gene. Data are expressed as means ± SEM (n=5). Asterisks indicate significant differences from the controls (P<0.01).
and sinusoids is filled with young mesenchymal cells, which proliferate very actively and sequentially [26]. The Igf2 gene is well known as a major regulator of fetal growth [27–29] and hematopoietic stem cell differentiation [30, 31]. IGF-II enhances the number of fetal liver stromal cells and supports in vitro erythroid colony formation [29, 32–34]. Dlk1 is relevant to fetal lethality and growth retardation in maternal disomy mice and null mutant mice [35–37]. The Dlk1 gene encodes a cell-surface transmembrane and secreted protein that is essential for normal hematopoiesis and erythropoietic differentiation [18, 19, 38–40]. Moore et al. [39] demonstrated that Dlk1 is activated in a cell line derived from the fetal liver, in which HSCs reproduce, and they defined a novel molecular pathway for stem cell regulation in the hematopoietic microenvironment of the fetal liver.

Erythroid differentiation in the liver of the ngw/Fg BMEs was apparently retarded and accompanied by a significant increase in immature blood cells, and the Igf2 and Dlk1 genes were repressed. The present results strongly suggest that the hematopoietic defects are a major cause of death before E13.5 in the ngw/Fg BMEs. Our series of studies revealed that ngw/Fg and ngw/Fg BMEs harboring mutations that allow the imprinted expression of Igf2 and Dlk1 genes, respectively, develop to term; however, both BME genotypes exhibit severe growth retardation and die soon after birth. The present results suggest that such dramatic extended development was caused by the effective amelioration of erythroid differentiation caused by expression of Igf2 and/or Dlk1. Together with the results of previous reports, these results suggest that Igf2 and Dlk1 are major contributors to fetal hematopoiesis and that the hematopoietic disorders are involved in developmental arrest of ngw/Fg BMEs at E12.5.

Understanding the mechanism by which Igf2 and Dlk1 facilitate fetal hematopoiesis is of interest. This study provided evidence that ngw/fg BMEs result in congenital aplasia of hematopoiesis in the liver, in which the proportion of immature erythroblasts increases, indicating a decrease in the expression of erythroid hematopoietic marker genes. Such hematopoietic defects in the fetal liver might be caused by disorders associated with the hematopoietic microenvironment, which is necessary for HSCs in ngw/fg BMEs. It has been reported that the cell populations found in the fetal liver microenvironment are associated with the development of hematopoiesis in mice [41], indicating that a mixed population of cells with mixed endodermal and mesodermal features corresponds to the hematopoietic-supportive fetal liver stroma. Igf2 and Dlk1, which are expressed in the stromal cells of the fetal liver, may be involved in proliferation of stromal cells and microenvironment formation. Based on these findings, we concluded that the hematopoietic defects in ngw/fg BMEs are due to the cessation of Igf2 and/or Dlk1 expression; this results in developmental arrest at E13.5 in ngw/fg BMEs. We can not rule out the possibility that other genes located on chromosome 12, such as Rht1 or Dio3, which are respectively known as a retrotransposon-like gene, with an open reading frame of unknown function and a negative regulator of thyroid hormone metabolism, might be involved in fetal hematopoiesis. However, the particular roles of both these genes in fetal hematopoiesis are completely unknown at present. Furthermore, global gene expression analysis by cDNA microarray revealed that Igf2 and Dlk1 play pivotal roles to improve the development of ngw/fg BMEs [9]. Thus, the present study suggests that Igf2 and Dlk1 predominantly function in a synergistic manner, because either gene can significantly improve the fetal liver hematopoiesis in ngw/Fg and ngw/Fg BMEs, respectively.

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