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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 ng<sup><small>ch7</small></sup>/fg BMEs contained increased numbers of immature c-kit<sup>+</sup>/ter119<sup>−</sup> hematopoietic cells, while the numbers of mature c-kit<sup>+</sup>/ter119<sup>+</sup> 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 the ng allele. We demonstrated that both genotypes of ng<sup><small>Δ</small></sup> and/or fg BMEs using ng oocytes harboring deletion of differentially methylated regions at distal chromosomes 7 and/or 12. The ng<sup><small>Δ</small></sup>/fg, ng<sup><small>Δ</small></sup>ch<sub>7</sub>/fg and ng<sup><small>Δ</small></sup>Double/fg BMEs using ng oocytes harboring deletion of differentially methylated regions at distal chromosomes 7 and/or 12. The ng<sup><small>Δ</small></sup>/fg, ng<sup><small>Δ</small></sup>ch<sub>7</sub>/fg and ng<sup><small>Δ</small></sup>Double/fg BMEs, respectively, express *Igf2*, *Dlk1* and both, and these embryos developed to term with specific phenotypes; the ng<sup><small>Δ</small></sup>/fg and ng<sup><small>Δ</small></sup>ch<sub>7</sub>/fg BMEs develop to term with severe growth retardation, and the ng<sup><small>Δ</small></sup>Double/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 ng<sup><small>Δ</small></sup>ch<sub>7</sub>/fg, ng<sup><small>Δ</small></sup>ch<sub>12</sub>/fg and ng<sup><small>Δ</small></sup>Double/fg BMEs were considerably restored, and particularly in the ng<sup><small>Δ</small></sup>Double/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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Due to imprinted genes, which exhibit monoallelic expression depending upon parental origin-dependent epigenetic modifications, such as DNA methylation imposed during gametogenesis, mouse parthenogenetic embryos never develop beyond day 10 of gestation in mice [1–4]. However, reconstructed bi-maternal embryos (ng/fg BMEs), which contain 2 haploid sets of genomes obtained from a non-growing stage oocyte (ng) and a fully grown oocyte (fg), develop to embryonic day (E) 13.5 [5]. A detailed gene expression analysis revealed that this extended development of ng/fg BMEs results from the appropriate expression of a majority of the imprinted genes: however, the paternally-methylated and paternally-expressed genes are still repressed [6, 7]. To induce expression of *Igf2* and *Dlk1* from the ng oocyte allele, we reconstructed ng/fg BMEs using ng oocytes harboring a deletion in the *H19* transcription unit and its differentially methylated region (*Δch7*) or a deletion in the methylated region IG-DMR (*Δch12*) in the ng allele. We demonstrated that both genotypes of ng<sup><small>Δch7</small></sup>/fg BMEs [7] and ng<sup><small>Δch12</small></sup>/fg BMEs [8] developed to E18.5, but also that they experienced severe growth retardation and died soon after recovery from the uterus. Furthermore, we recently demonstrated that ng/fg BMEs harboring both Ach7 and Δch12 (*ΔDouble*) expressed *Igf2* and *Dlk1* at appropriate levels and developed to normal adults at high frequency [9]. From these results, it has been suggested that both *Igf2* and *Dlk1* are critical for normal mouse embryonic development, while these paternally expressed imprinted genes function as a strict barrier to development beyond mid-gestation (E13.5) in mice [7, 8, 10].

However, phenotypical analyses for ng/fg bi-maternal fetuses have rarely been reported. In order to gain a further understanding of the features of the bi-maternal fetuses, we focused on hematopoiesis because the fetal hematopoietic system in the liver is critically important for survival and growth of mouse embryos. In mouse embryos, fetal hematopoietic systems dynamically change during development; the yolk sac (YS) [11], aorta- gonad-mesonephros (AGM) [12–14] and placenta (PL) [15] have been reported as the origins of hematopoietic stem cells (HSCs) during early gestation. Beginning in mid-gestation, the fetal liver, in which hematopoietic stem cells (HSCs) relocate from multiple sources and colonize, functions as a major hematopoeitic organ, and subsequently, hematopoiesis permanently occurs in the bone marrow throughout life [16]. Interestingly, it is known that the *Igf2* and *Dlk1* genes are involved in fetal hematopoiesis in the fetal liver. The *Igf2* gene, which encodes a growth-promoting factor insulin-like growth factor 2 (IGF-II), plays a potential role for the growth of fetal and hematopoietic stem cells [17]. *Dlk1* encodes delta-like homolog 1, a cell-surface transmembrane secreted protein belonging to the epidermal growth factor-like homoeoctic family that also appears to play significant roles for normal hematopoiesis [18, 19].

In the present study, we conducted histological and gene expres-
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 in 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 \(\mu\)M 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 \(\mu\)M 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 SrCl\(_2\) in Ca\(^{2+}\)-free M16 medium for 2 h. These embryos were cultured in M16 medium for 3.5 days in an atmosphere of 5% CO\(_2\), 5% O\(_2\) and 90% N\(_2\) 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 mice of mouse BMEs (ng\(^{wt}/fg\), ng\(^{Δch7}/fg\), ng\(^{Δch12}/fg\) and ng\(^{ΔDouble}/fg\)) and control embryos (wt) at day 12.5 of gestation were fixed overnight in 4% 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 at 4°C. The livers were incubated in 10–20% graded sucrose and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan). Sections (10 \(\mu\)m) of the liver tissues were placed on the slides, and ISH was performed essentially as described previously. Digoxigenin-labeled RNA probes 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'—TTG TTG CTC GGG CTG CTG A-3') and Dlk1 (primers: 5'—GAT GTG TTG CTC GGG ATG AAG GAA-3', and 5'—GGT GAG GAC AAG CTT TTT TTT C-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 (ngΔch12/fg, ngΔch7/fg and ngΔch12/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 × 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 ngΔch12/fg BMEs. The number of parenchyma 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 ngΔch12/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 ngΔch12/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 ngΔch12/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 liver, 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 hematopoiesis, 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 erythroids (Fig. 3A). In contrast, the differentiation of erythroids was limited in the ngΔch12/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 ngΔch12/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, EkIf and NF-2E genes was evidently decreased in the ngΔch12/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 EkIf 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 ngΔch12/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 livers (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 ngΔch12/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-
WU et al. 180

ders in the liver. Experiments involving introduction of \(Igf2\) and/or \(Dlk1\) 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 \(Igf2\) [20–24] and \(Dlk1\) [18, 19, 25] 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

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**Fig. 1.** 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), intrhepatocyte 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.

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**Fig. 2.** Expression of the \(Igf2\) and \(Dlk1\) genes in the liver at E12.5. (A) \(Igf2\) and \(Dlk1\) 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 \(Actb\) 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) \(Igf2\) and \(Dlk1\) 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.

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**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) \(GATA1\), \(EpoR\), \(EKLF\) 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 \(Actb\) gene. Data are expressed as means ± SEM (n=5). Asterisks indicate significant differences from the controls (P<0.01).
involved in proliferation of stromal cells and microenvironment which are expressed in the stromal cells of the fetal liver, may be of interest. This study provided evidence that the liver, in which the proportion of immature erythroids increases, marker genes. Such hematopoietic defects in the fetal liver might be caused by disorders associated with the hematopoietic microenvironment of the fetal liver.

Erythroid differentiation in the liver of the ng\(^{wt}\)/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 ng\(^{wt}\)/fg BMEs. Our series of studies revealed that ng\(^{ch7/fg}\) and ng\(^{ch12/fg}\) 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 due to 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 ng\(^{wt}\)/fg BMEs at E12.5.

Understanding the mechanism by which Igf2 and Dlk1 facilitate fetal hematopoiesis is of interest. This study provided evidence that ng\(^{wt}\)/fg BMEs result in congenital aplasia of hematopoiesis in the liver, in which the proportion of immature erythrocytes 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 ng\(^{wt}\)/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 ng\(^{wt}\)/fg BMEs are due to the cessation of Igf2 and/or Dlk1 expression; this results in developmental arrest at E13.5 in ng\(^{wt}\)/fg BMEs. We can not rule out the possibility that other genes located on chromosome 12, such as Rtl1 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 ng\(^{wt}\)/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 ng\(^{ch7/fg}\) and ng\(^{ch12/fg}\) BMEs, respectively.

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