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—Full Paper—

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

Qiong WU[1)#], Manabu KAWAHARA[1)] and Tomohiro KONO[1)]

[1)]*Department of BioScience, Tokyo University of Agriculture, Tokyo 156-8502, Japan*
[#]*Present: Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, China*

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, *Igf*2 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^{wt}/fg BMEs contained increased numbers of immature c-kit^+/$ter119^-$ hematopoietic cells, were 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 *Igf*2 and *Dlk1* expression was repressed in the liver. To understand the role of paternally-methylated imprinted genes on chromosomes 7 and 12, particularly *Igf*2 and *Dlk1*, in fetal liver hematopoiesis, we constructed $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/fg BMEs using ng oocytes harboring deletion of differentially methylated regions at distal chromosomes 7 and/or 12. The $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/fg BMEs, respectively, express *Igf*2, *Dlk1* and both, and these embryos developed to term with specific phenotypes; the $ng^{\Delta ch7}$/fg and $ng^{\Delta ch12}$/fg BMEs develop to term with severe growth retardation, and the $ng^{\Delta Double}$/fg BMEs can survive to become normal female adults. By inducing *Igf*2 and *Dlk1* expression, the proportions of mature and immature hematopoietic cells in the livers of the $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/fg BMEs were considerably restored, and particularly in the $ng^{\Delta Double}$/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 impringing, 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 $ng^{\Delta ch7}$/fg BMEs [7] and $ng^{\Delta ch12}$/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 Δch7 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 hematopopietic 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 homeotic family that also appears to play significant roles for normal hematopoiesis [18, 19].

In the present study, we conducted histological and gene expres-

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Correspondence: T. Kono (e-mail: tomohiro@nodai.ac.jp)

Table 1. Primers used for quantitative gene expression analysis by real time PCR

Genes	Primer sequences	GenBank accession No.
Igf2	5'-AGGGGAGCTTGTTGACACG-3'	NM-010514
	5'-GGGTATCTGGGGAAGTCGTC-3'	
Dlk1	5'-ACTTGCGTGGACCTGGAGAA-3'	NM-010052
	5'-CTGTTGGTTGCGGCTACGAT-3'	
GATA1	5'-CTC TAC CCT GCC TCA ACT G-3'	NM-008089
	5'-TTG CTG ACA ATC ATT CGC TT-3'	
EpoR	5'-TTCACCCAACGCTTGGAAGAC-3'	NM-010149
	5'-ATGCGGTGATAGCGAGGAGAA-3'	
EKLF	5'-CGGGAGAGAAGCCTTATGCC-3'	NM-010635
	5'-GGTGACGCTTCATGTGCAGA-3'	
NF-2E	5'-ACGTGGACATGTACCCAGTGG-3'	NM-008685
	5'-GCCACCTTGTTCTTGCCCCGT-3'	
GAPDH	5'-GTCGTGGAGTCTACTGGTGTC-3'	NM-199472
	5'-GAGCCCTTCCACAATGCCAAA-3'	

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, $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/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 cultured for 14 h in α-MEM medium (GIBCO, Grand Island, NY, USA) containing 5% calf serum. A spindle from the reconstructed oocytes was again transferred into 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 livers of mouse BMEs (ng^{wt}/fg, $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/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 day12.5 of gestation, the livers of the BMEs (ng^{wt}/fg, $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/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* (primesr: 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, $ng^{\Delta ch7}$/fg, $ng^{\Delta ch12}$/fg and $ng^{\Delta Double}$/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 (ng^{wt}/fg, ng$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/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 ng^{wt}/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 ng^{wt}/fg BMEs (Fig. 1B). These prominent disorders were obviously restored in the livers of ng$^{\Delta ch7}$/fg, ng$^{\Delta ch12}$/fg and ng$^{\Delta Double}$/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 liver cells might be involved in restricted development of ng^{wt}/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 ng$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/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 ng$^{\Delta Double}$/fg BME livers were expressed at the same levels as those in the wt livers. In the ng^{wt}/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 ng$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/fg livers, respectively. In the ng$^{\Delta Double}$/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 ng^{wt}/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 ng^{wt}/fg BMEs compared with that in the wt cells, *i.e.*, 16% *vs.* 10%. This disorder in erythroid differentiation was significantly restored in the ng$^{\Delta ch7}$/fg, ng$^{\Delta ch12}$/fg and ng$^{\Delta Double}$/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, ng$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/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 ng$^{\Delta ch7}$/fg, ng$^{\Delta ch12}$/fg and ng$^{\Delta Double}$/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 ng^{wt}/fg and ng$^{\Delta ch7}$/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 ng$^{\Delta ch7}$/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 ng$^{\Delta ch12}$/fg BME livers but did not recover to the levels in the wt livers. The expression of the erythroid hematopoietic markers in the ng$^{\Delta Double}$/fg BMEs 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 ng^{wt}/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-

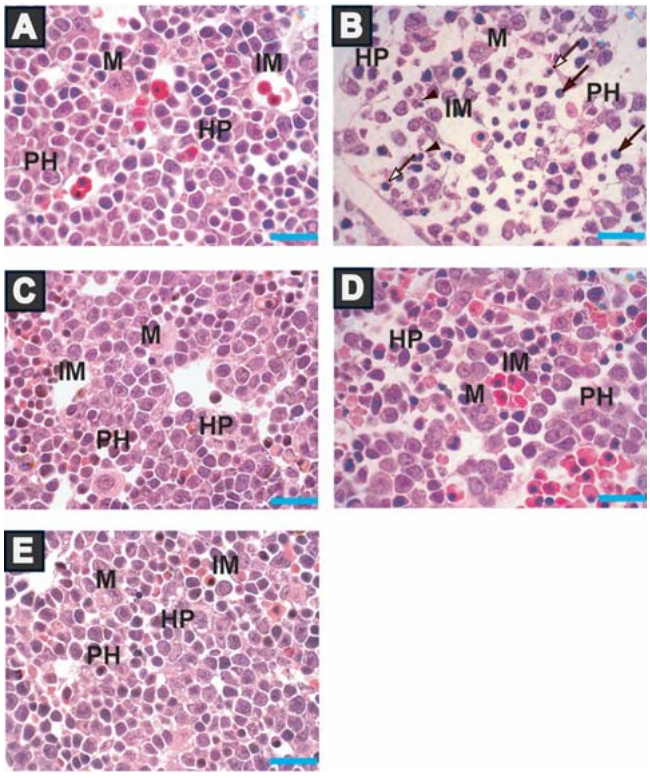

Fig. 1. Histological examination of the fetal livers. Sections of the fetal livers obtained from (wt) mice (A) and the 4 BMEs, ng^{wt}/fg (B), ng$^{\Delta ch7}$/fg (C), ng$^{\Delta ch12}$/fg (D) and ng$^{\Delta Double}$/fg (E), at E12.5 stained with hematoxylin and eosin (HE). Developing parenchymal 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.

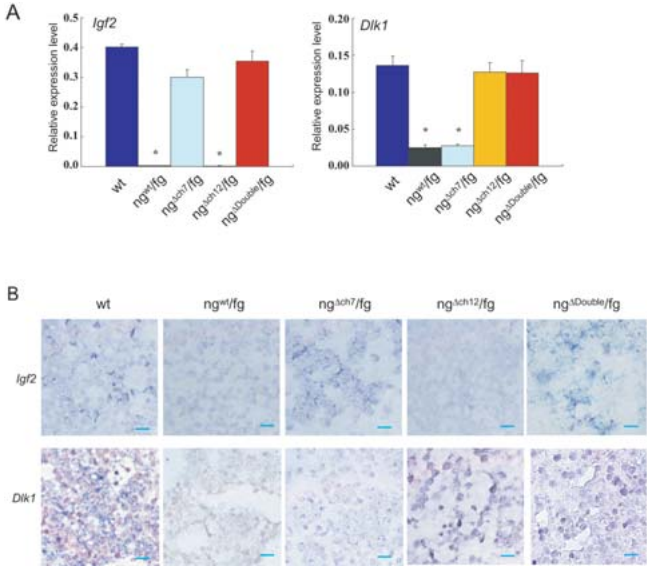

Fig. 2. Expression of the *Igf2* and *Dlk1* genes in the liver at E12.5. (A) Igf2 and Dlk1 expression were quantitatively analyzed by real-time PCR. The expression of each gene is shown as the average of the expression levels relative to those of the *Actb* gene, which was used as an internal control. 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.

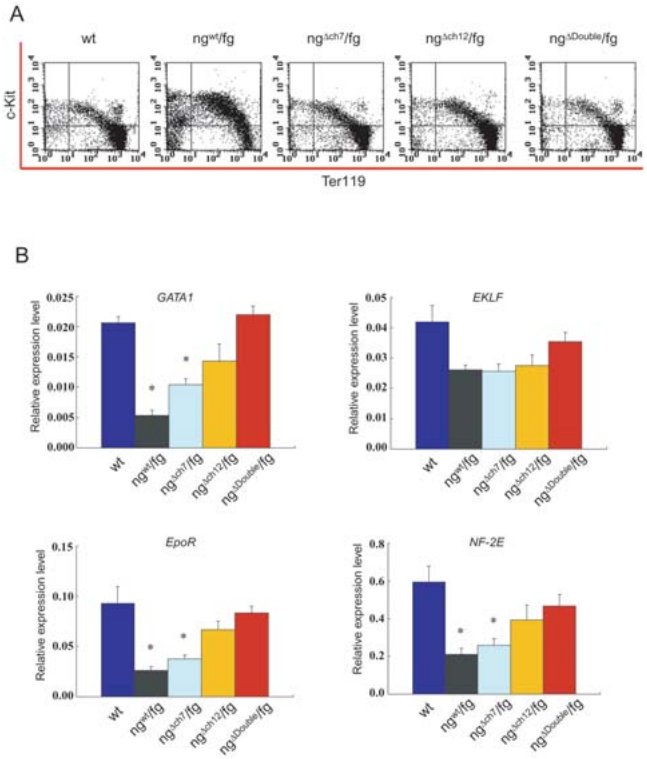

Fig. 3. Definitive erythropoiesis in the fetal liver of the ng^{wt}/fg, ng$^{\Delta ch7}$/fg, ng$^{\Delta ch12}$/fg and ng$^{\Delta Double}$/fg BMEs at E12.5. (A) FACS analysis of the 3 BME livers cells at E12.5. Single fetal liver cells obtained from a wt mouse and BMEs were immunostained with anti-Ter119 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).

ders in the liver. Experiments involving introduction of *Igf2* and/or *Dlk1* expression in mutant BMEs clearly 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

Table 2. Proportion of fetal liver hematopoietic cells expressing Ter119 and/or C-kit

	c-Kit$^+$/Ter119$^-$	c-Kit$^+$/Ter119$^+$	c-Kit$^-$/Ter119$^-$	c-Kit$^-$/Ter119$^+$
wt	10 ± 0.8	15 ± 0.4	8 ± 1.3	67 ± 1.5
ng^{wt}/fg	16 ± 0.3	42 ± 7.6	7 ± 1.4	35 ± 6.6
ng$^{\Delta ch7}$/fg	11 ± 2.5	16 ± 3.7	11 ± 2.5	62 ± 3.1
ng$^{\Delta ch12}$/fg	9 ± 0.8	24 ± 0.6	6 ± 0.8	61 ± 0.6
ng$^{\Delta Double}$/fg	11 ± 1.3	15 ± 1.6	10 ± 1.4	65 ± 1.5

Values represent means ± SD. About 10,000 cells were counted in each sample, and the analysis was repeated 3 times.

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 erythroid 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 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$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/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 erythroids 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 microenvironmental 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/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$^{\Delta ch7}$/fg and ng$^{\Delta ch12}$/fg BMEs, respectively.

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