Journal of Reproduction and Development, Vol. 56, No. 1, 2010

—Full Paper—

Involvement of Insulin-like Growth Factor 2 in Angiogenic Factor Transcription in Bi-maternal Mouse Conceptuses

Manabu KAWAHARA[1], Qiong WU[2]# and Tomohiro KONO[2]

[1]*Laboratory of Animal Resource Development, Faculty of Agriculture, Saga University, Saga 840-8502 and* [2]*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. Imprinted genes in which only one of the two parental chromosome copies is expressed have a substantial effect on mammalian ontogenesis. On mouse distal chromosome 7, the paternally expressed gene insulin-like growth factor 2 (*Igf2*) is separated by approximate 100 kb from the maternally expressed non-coding gene *H19*. However, there is limited knowledge of the manner in which *Igf2* transcription affects the other genes involved in embryonic development. To clarify this, we performed quantitative gene expression analysis for representative angiogenic factors—*Vegf, Flt1, Flt4, Flk1, Ang1, Ang2, Tie1*, and *Tie2*—for 3 types of bi-maternal conceptuses containing genomes with non-growing (ng) and fully grown (fg) oocytes. The genetic backgrounds of the ng oocytes were 1) the wild type (ng^{wt}), 2) mutant mice carrying a 3-kb deletion of the *H19* transcription unit ($ng^{H19\Delta3\text{-}KO}$/fg) and 3) mutant mice carrying a 13-kb deletion in the *H19* transcription unit, including the germline-derived differentially methylated region on chromosome 7 ($ng^{H19\Delta13\text{-}KO}$/fg). In the ng^{wt}/fg and $ng^{H19\Delta3\text{-}KO}$/fg placentae, *Vegf* and *Flt1* were upregulated compared with the mean value for the wt placenta, whereas in the $ng^{H19\Delta13\text{-}KO}$/fg placenta, these transcriptional levels were restored. In the fetus, however, only 2 genes among the 8 genes analyzed were significantly changed in the bi-maternal fetuses, indicating that the effects of the *Igf2* mRNA level on angiogenic factor transcription in the fetus differed from those in the placenta. Our results indicated that the *Igf2* mRNA level affects transcription of angiogenic factors in both bi-maternal placentae and fetuses.

Key words: Angiogenic factors, Mouse, *H19*, Insulin-like growth factor 2 (*Igf2*), Serial nuclear transfer

(**J. Reprod. Dev. 56:** 79–85, 2010)

Genomic imprinting, whereby certain genes exhibit parental origin-specific expression, is a critical control mechanism in embryonic development of mammals, as it inhibits parthenogenesis by ensuring that genes from both parents are functionally present. Therefore, genomic imprinting is essential for normal ontogeny, and imprinted genes are regulated by epigenetic modifications, including DNA methylation. Acquisition of methylation occurs in each parental germline during male or female gametogenesis [1, 2]. Most imprinted genes are regulated by maternally-derived methylation, while only three regions on mouse chromosomes 7, 9 and 12 have been identified as genes regulated by paternally-derived methylation [3].

We have previously analyzed mouse conceptuses derived from only maternal genomes that carry two sets of haploid genomes from non-growing (ng) and fully grown (fg) oocytes, which are able to develop to embryonic day (E) 13.5 [4–6]. In these embryos, the paternally expressed genes, *Peg1/Mest*, *Peg3* and *Snrpn*, were activated, while the maternally expressed genes, *Igf2r* and *p57Kip2*, were silent in the alleles derived from a non-growing oocyte genome [6, 7]. Hence, on theoretical grounds, all of the imprinted gene transcriptions regulated by maternal-methylation imprinting could be corrected in bi-maternal ng/fg embryos. However, paternally methylated imprinted genes containing the *H19* and *Igf2* genes have not been altered in the ng allele [4, 5, 8].

Chromosome 7 contains one protein-coding gene expressed by the paternally inherited chromosome, insulin-like growth factor 2 (*Igf2*); on the maternally inherited chromosome, this gene is repressed, and a non-coding RNA transcript, *H19*, is expressed (Fig. 1) [9–11]. The *Igf2* gene, which encodes a growth-promoting factor (IGF2), is well known as a primary regulator of fetal growth [12–14]. In ng^{wt}/fg embryos derived from wild-type ng oocytes, *H19* is biallelically expressed, while *Igf2* is repressed, as the enhancers that are shared by *Igf2* and *H19* act preferentially on *H19* on both chromosomes (Fig. 1) [5]. Furthermore, mutated ng oocyte genomes that mimic paternal function at chromosome 7 allow us to analyze bi-maternal ng/fg conceptuses after E13.5; specifically, one harbors a 3-kb deletion of the *H19* transcription unit ($H19^{\Delta3}$), and the other harbors a 13-kb deletion containing the differentially methylated region and the *H19* transcription unit ($H19^{\Delta13}$) [8, 15]. The $ng^{H19\Delta3\text{-}KO}$/fg bi-maternal conceptuses with monoallelic *H19* expression are able to develop to E17.5 with a leakage level of *Igf2* expression [15]; additionally, they show further extended development to E19.5 with corrected expression patterns for both the *H19* and *Igf2* genes (Fig. 1) [4, 8].

Nevertheless, the $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg bi-maternal conceptuses also form placentae concomitantly with a malformed labyrinthine layer and anomalous circulatory system [15, 16]. The labyrinth plays a crucial role in the exchange of nutrients, gases and waste between maternal and fetal blood, and its vasculature is one

Received: August 20, 2009
Accepted: October 1, 2009
Published online in J-STAGE: November 2, 2009

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

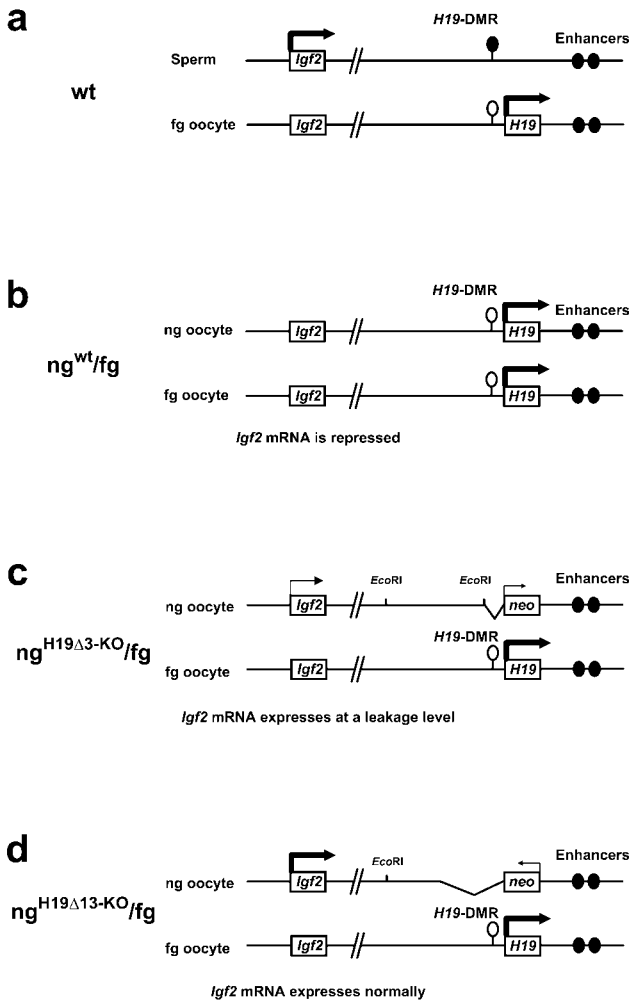

Fig. 1. Expression patterns of paternally methylated imprinted genes controlled by the *Igf2-H19* domain on chromosome 7 in mouse conceptuses. Wild-type conceptuses (wt; a) and 3 types of bi-maternal conceptuses, ng^{wt}/fg (b), $ng^{H19\Delta3\text{-}KO}$/fg (c) and $ng^{H19\Delta13\text{-}KO}$/fg (d).

of the most important factors supporting fetal viability. Histological analysis of the $ng^{H19\Delta3\text{-}KO}$/fg placenta has shown that severe necrosis occurs throughout the tissue, particularly in the labyrinthine layer [15]. Moreover, scanning electron microscopy studies of casts have definitively shown that in $ng^{H19\Delta13\text{-}KO}$/fg placentae, both the maternal sinusoids and fetal blood vessels within the labyrinth are defective [16]. However, the vasculature in the $ng^{H19\Delta13\text{-}KO}$/fg placenta is clearly improved compared with the ng^{wt}/fg placenta; the mechanism by which this occurs has yet to be elucidated fully.

Generally, IGF2 action can affect the growth of the fetus; overexpression of *Igf2* can increase the size at birth up to 160% [17] and the size at E17 up to 200% [18] in a dose-dependent manner. In human hepatocellular carcinomas cells, it is known that hypoxia-induced vascular endothelial growth factor (*VEGF*) expression [19] is increased by *IGF2*. *VEGF* plays a role in formation of new blood vessels from preexisting ones. The regulatory pathways for insulin-induced angiogenesis have been investigated in human umbilical venous endothelial cells [20], and *IGF2* may be implicated in several aspects of placental development, including blood vessel formation. Meanwhile, the targets of *H19* remain to be identified, although several studies have been conducted to understand the biological function of *H19* mRNA using mutant mice harboring the deletion of the *H19* transcription unit [13, 21] and overexpressing *H19* mRNA [22]. To date, there has been no definite evidence that *H19* transcription is positively involved in vascular formation during the developmental period in mice.

In this study, to clarify the contributions of *Igf2* mRNA expression to mouse ontogenic angiogenesis, we conducted a thorough investigation of the transcriptional levels of 8 representative angiogenic factors—*Vegf, Flt1, Flt4, Flk1, Ang1, Ang2, Tie1* and *Tie2*—in 3 bi-maternal conceptus models, the ng^{wt}/fg, $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg genotypes. These bi-maternal conceptuses exhibit varying *Igf2* mRNA expression levels. In ng^{wt}/fg conceptuses, *Igf2* mRNA expression is repressed, whereas the $ng^{H19\Delta3\text{-}KO}$/fg conceptuses exhibit *Igf2* mRNA expression at a leakage level [5, 15]. On the other hand, the *Igf2* transcription levels in the $ng^{H19\Delta13\text{-}KO}$/fg conceptuses are close to wild-type levels [8]. *Vegf* is a potent inducer of angiogenesis. This is necessary for tissue development and promotes tumor growth [23, 24]. *Flt1, Flt4* and *Flk1* are a set of mammalian tyrosine kinase receptors for *Vegf* that exhibit important regulatory functions in the formation of new blood vessels during embryonic vasculogenesis [24, 25]. The angiopoietin ligand (*Ang1* and *Ang2*) and Tie receptor families (*Tie1* and *Tie2*) are also important regulators of blood vessel growth, maturation, and function [26]. We report here that the transcription levels of the *Igf2* gene affect the mRNA expression levels of these angiogenic factors.

Materials and Methods

Production of ng/fg bi-maternal conceptuses

Serial nuclear transfer was carried out as outlined previously [27]. Ng oocytes ($ng^{H19\Delta3\text{-}KO}$/fg or $ng^{H19\Delta13\text{-}KO}$/fg) containing a haploid set of genome $H19^{\Delta3}$ or $H19^{\Delta13}$ mutant females, as well as a genome obtained from ovulated MII oocytes of wild-type B6D2F1 mice, were constructed by nuclear transfer. Fusion of the diplotene oocytes with enucleated oocytes at the germinal vesicle stage (GV) was induced with an inactivated Sendai virus (HVJ, 2700 hemagglutinating activity units/ml). After fusion, the reconstituted oocytes were cultured for 14 h in α-MEM medium (GIBCO, Grand Island, NY, USA). The GV oocytes were manipulated in a medium containing 200 μM dbcAMP and 5% calf serum throughout the experiment and were released from the medium 1 h after fusion with a non-growing stage oocyte. A set of MII chromosomes from reconstituted oocytes was transferred into an enucleated MII oocyte that had been collected from the oviducts of superovulated B6D2F1 mice 15–16 h after hCG injection. ng^{wt}/fg bi-maternal embryos were also produced from wild-type B6D2F1 mice as described above. The reconstructed oocytes were artificially activated 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%

Table 1. Primer sequences used for real-time PCR

Gene	Forward	Reverse	Annealing temperature (C)	Size of PCR products (bp)
Igf2	aggggagcttgttgacacg	gggtatctggggaagtcgtc	59	225
H19	catgtctgggcctttgaa	ttggctccaggatgatgt	55	245
Vegf	tggctttactgctgtacctc	tggtgatgttgctctctgac	58	273
Flt1	agactcttgtcctcaactg	gaggtgttgaaagactgga	55	238
Flt4	agaagctggttttgaactg	caatgacctctgtgctttc	55	243
Flk1	tcgctctgtggttctgcgtg	ggtttgaaatcgaccctcgg	65	450
Ang1	gagataggaaccagtctct	taagttgcttctccaactc	53	328
Ang2	gaagaaggagatggtggag	ggggaaggtcagtgtgtaga	61	595
Tie1	taggcgtcttctcctgtgt	ggtattgaagtaggatccgttgt	58	203
Tie2	gtccgagctagagtcaacac	gagctggtactgagtaacgg	58	261
Gapdh	gtcgtggagtctactggtgtc	gagcccttccacaatgccaaa	60	240

CO_2, 5% O_2 and 90% N_2 at 37 C [28]. The embryos that developed to the blastocyst stage were transferred into the uterine horns of recipient female mice at 2.5 days of pseudopregnancy. The fetuses and placentae were recovered from the pregnant mice at E12.5 and used in the subsequent analyses as in our previous studies [16, 29].

Quantitative gene expression analysis

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN, Tokyo, Japan) from four types of whole fetuses and placentae, wild-type (wt; n=3) and bi-maternal conceptuses derived from ng^{wt}/fg (n=5), $ng^{H19\Delta3\text{-}KO}$/fg (n=5) and $ng^{H19\Delta13\text{-}KO}$/fg (n=4) embryos. Using ISOGEN (Nippon Gene, Tokyo, Japan), total RNA was isolated from the fetuses and placentae at E12.5. After total RNA was treated with DNase (Promega, Madison, WI, USA) to exclude the genomic DNA, the absence of genomic DNA contamination was confirmed by the lack of amplification of *GAPDH* by PCR. The cDNAs were then synthesized using a SuperScript™ II RnaseH reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) in a reaction solution (20 μl) containing total RNA (1 μg) prepared from each placenta. Finally, quantitative analysis of gene expression was performed using real-time PCR (LightCyclerTM System, Roche Molecular Biochemicals, Mannheim, Germany) after preparing the reaction mixture (LightCycler FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals). The primers used for the analysis are listed in Table 1.

Statistical analyses

Statistical analyses of all data for comparison to wild-type controls were carried out using one-way analysis of variance (ANOVA) and the Fisher PLSD test using the statistical analysis software StatView (Abacus Concepts, Berkeley, CA, USA). A P value of <0.05 was considered statistically significant.

Results

Confirmation of the mRNA expression levels of Igf2 and H19 in bi-maternal conceptuses

To confirm the mRNA expression levels of *Igf2* and *H19* in the bi-maternal conceptuses used in this study, we first analyzed the mRNA levels of these two genes in individual wt, ng^{wt}/fg,

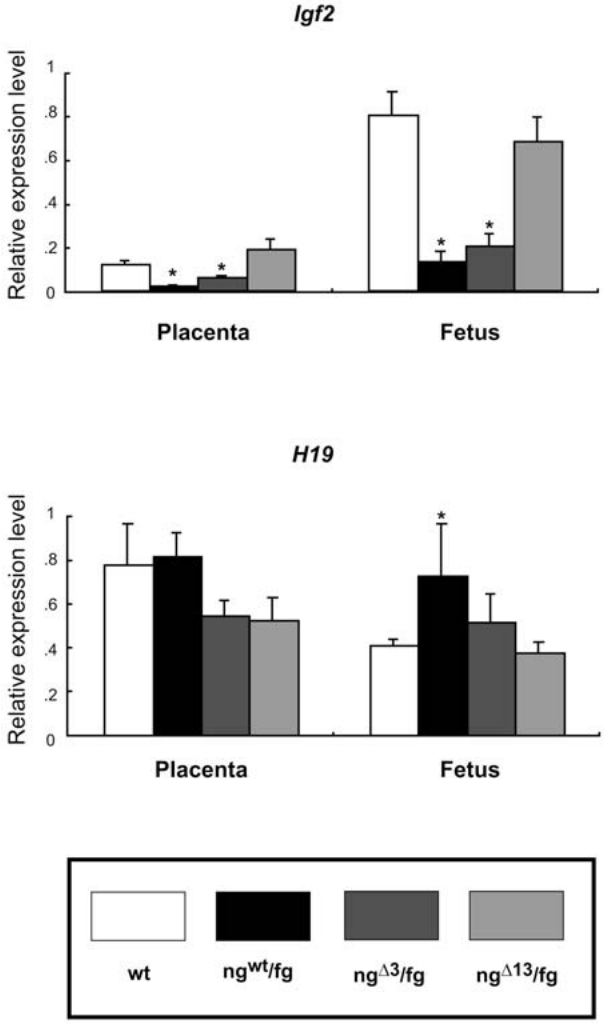

Fig. 2. The mRNA expression levels of *Igf2* and *H19* genes in the bi-maternal conceptuses: comparison of the 3 types of bi-maternal conceptuses, ng^{wt}/fg, $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg, with the wild type. Values are means ± SEM and levels of expression relative to that of the internal control gene (*Gapdh*).
* Significant differences compared with wt placentae (P<0.05).

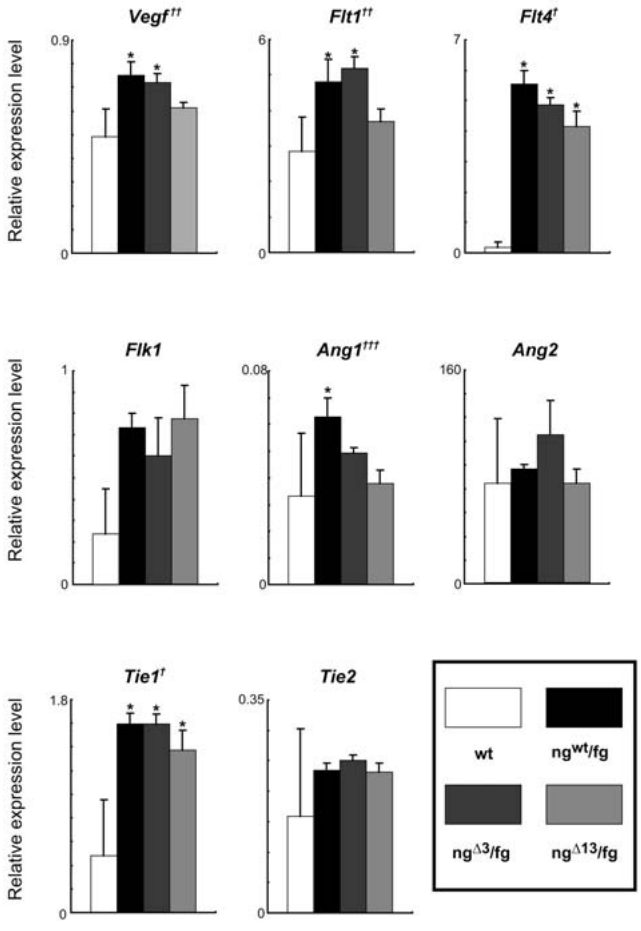

Fig. 3. Graphical representations of the transcription levels of angiogenic factors in the bi-maternal placenta. The transcription levels of eight genes for representative angiogenic factors, *Vegf; Flt1, Flt4, Flk1, Ang1, Ang2, Tie1* and *Tie2*, in 12.5-day bi-maternal placentae were analyzed using quantitative real-time PCR. Values represent levels of expression relative to the internal control gene (Gapdh). †† transcription restored in the $ng^{H19\Delta13\text{-}KO}$/fg placenta, † transcription not corrected in all of the bi-maternal placentae. ††† transcription restored in the $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg placentae. Values represent means ± SEM. * Significant differences compared with wt placentae ($P<0.05$).

$ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg fetuses and placentae (Fig. 2). As expected, the results were consistent with our previous reports in regard to the *Igf2* mRNA levels; specifically, the expressions of both genes were repressed in the ng^{wt}/fg conceptuses and expressed at leakage levels in the $ng^{H19\Delta3\text{-}KO}$/fg conceptuses [5, 15]. In the $ng^{H19\Delta13\text{-}KO}$/fg conceptuses, the *Igf2* mRNA levels were fully restored [8, 16]. However, the *H19* gene was not theoretically expressed in all of the bi-maternal conceptus types. We do not have a clear explanation for this finding at the moment; however, the quantitative expression analysis was carried out correctly. We have observed a smilar phenomenon, a lack of theoretical expression of non-coding RNAs such as *H19*, in previous studies [8, 16, 29, 30]. However, the results confirm that these bi-maternal conceptuses can be used to explore the effects of *Igf2* mRNA levels on transcription of the other genes in the mouse placenta and fetus.

Transcription levels of angiogenic factors in bi-maternal placenta

We performed quantitative gene expression analysis for the *Vegf, Flt1, Flt4, Flk1, Ang1, Ang2, Tie1* and *Tie2* genes in individual wt, ng^{wt}/fg, $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg placentae using real-time PCR (Fig. 3). There were no significant differences in *Flk1, Ang2* and *Tie2* transcription between any of the bi-maternal placentae or the wt placenta. The expressions of *Vegf* and *Flt1* were corrected in the $ng^{H19\Delta13\text{-}KO}$/fg placenta, but not in the other placentae (Fig 3; †† double daggers). In the ng^{wt}/fg and $ng^{H19\Delta3\text{-}KO}$/fg placentae, both of these RNAs were upregulated compared with the mean values in the wt placenta. The *Flt4* and *Tie1* RNA expression levels remained elevated in all of the bi-maternal placentae (Fig. 3; † single dagger). Interestingly, *Ang1* RNA expression was corrected in the $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg placentae, but not in the ng^{wt}/fg placenta. Thus, we confirmed that alteration of the *Igf2* mRNA expression level can affect transcription levels of angiogenic factors in mouse placentae.

Transcription levels of angiogenic factors in the bi-maternal fetus

To gain further understanding of the transcription levels of angiogenic factors in bi-maternal conceptuses, we also carried out quantitative gene expression analysis in fetuses, as was conducted for the placenta (Fig. 4). Unlike the placenta, *Vegf* mRNA expression in the fetus was upregulated only in the ng^{wt}/fg conceptus (Fig. 4; ††† triple daggers). The *Flt1* transcription level in the fetus was also elevated in the ng^{wt}/fg and $ng^{H19\Delta3\text{-}KO}$/fg conceptuses (Fig. 4; † single dagger). However, the transcription levels of *Flt4, Ang1* and *Tie1* in all the bi-maternal fetuses were not statistically different from those in the wt fetus. These results suggest that, in the bi-maternal fetus, the *Igf2* mRNA expression level may affect the transcription levels of angiogenic factors via mechanisms distinct from those in the placenta.

Discussion

While the embryonic vascular system develops in anticipation of the demands of the growing embryo for oxygen and nutrients, angiogenesis in the organism occurs in response to the metabolic requirements of tissues and is efficiently triggered by hypoxia [24]. A variety of diseases are associated with either insufficient or excess blood vessel growth. Furthermore, adaptation of the maternal uterine vasculature is also essential for normal fetal and placental development, during which angiogenesis is considered one of the most critical adaptive changes. The process of placental vasculogenesis/angiogenesis requires tight orchestration between vascular endothelial cell-specific ligands and their tyrosine kinase receptors. These include the *Vegf* family members, including placental growth factor and the angiopoietins, primarily *Ang1* and *Ang2* [31]. Lack of appropriate function of one or more of these molecules and/or their receptors may adversely affect pregnancy outcome. Meanwhile, genes undergoing the phenomenon of

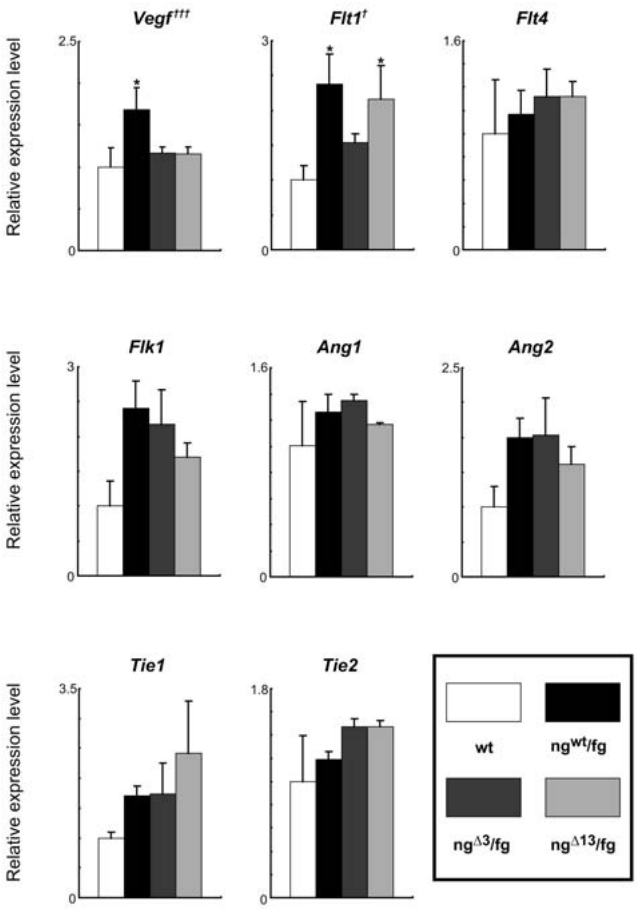

Fig. 4. Graphical representations of the transcription levels of angiogenic factors in bi-maternal fetuses. The transcription levels of eight genes for representative angiogenic factors, *Vegf; Flt1, Flt4, Flk1, Ang1, Ang2, Tie1* and *Tie2,* in 12.5-day bi-maternal fetuses were analyzed using quantitative real-time PCR. Values represent levels of expression relative to the internal control gene (Gapdh). † transcription not corrected in the ng^{wt}/fg and $ng^{H19\Delta13\text{-}KO}$/fg bi-maternal fetuses, ††† transcription restored in the $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg fetuses. Values represent means ± SEM. * Significant difference compared with wt fetuses ($P<0.05$).

genomic imprinting are also involved in regulation of major functions at the feto-maternal interface, such as nutrient transport, trophoblast proliferation, invasion and angiogenesis [32–34]. We have previously observed that vasculature in bi-maternal placentae with corrected imprinted genes regulated by the *Igf2-H19* domain on chromosome 7 is relatively improved compared with bi-maternal placentae that lack the ability to mimic the expression pattern of the paternal chromosome [16]. However, there is only a limited understanding of the manner in which the *Igf2* mRNA expression level contributes to vasculogenesis/angiogenesis in mouse conceptuses.

Propagating effects of imprinted genes controlled by the Igf2-H19 domain on angiogenic factor transcription in the placenta

The present study demonstrates that transcription levels of angiogenic factors in the bi-maternal mouse placenta can be affected in an *Igf2* mRNA level-dependent manner. Only $ng^{H19\Delta13\text{-}KO}$/fg placenta from among the bi-maternal placentae analyzed had the *Vegf* and *Flt1* mRNA expression levels that were corrected to those of the wild-type placenta, suggesting that appropriate transcription levels of these angiogenic factors are moderately dependent on the *Igf2* mRNA expression level in the placenta (Figs. 3 and 5). In contrast, the $ng^{H19\Delta3\text{-}KO}$/fg placenta with a leakage level of *Igf2* mRNA expression exhibited anomalously elevated *Vegf* and *Flt1* mRNA levels. Although the direct association between *Igf2* and such angiogenic factors is not fully understood, we have previously observed impaired vasculogenesis in the ng^{wt}/fg placenta and the $ng^{H19\Delta3\text{-}KO}$/fg placenta [15, 16]. In these bi-maternal placentae, severe ischemic necrosis was observed throughout the placental tissues, suggesting that this placental defect causes hypoxidosis and metabolic defects. Our results imply that placental hypoxia occurs in bi-maternal conceptuses when *Igf2* mRNA is not sufficiently expressed.

Hypoxia is known to be a potent stimulus for expression of *Vegf* and *Flt1*, and these mRNAs have been shown to be upregulated by hypoxia in a variety of cell types; their increase is thought to be a major mechanism triggering hypoxia-induced angiogenesis [35–37]. However, the growth of capillaries in the $ng^{H19\Delta3\text{-}KO}$/fg placenta was not sufficient to completely recuperate from hypoxia symptoms, and therefore, *Flt4* mRNA was overexpressed under hypoxic conditions in all of the bi-maternal placentae, suggesting that appropriate transcription from paternally methylated imprinted genes on chromosome 12 is required to complete definitive placentation [16, 38]. *Tie1* mRNA was also overexpressed in all the bi-maternal placentae.

The *angiopoietin-Tie2* system also plays a key role in vessel remodeling, maturation and stabilization in vasculogenesis/angiogenesis. *Tie1* and *Tie2* belong to a subfamily of vascular tyrosine kinase receptors expressed predominantly in endothelial cells, whose ligands are *Ang1* and *Ang2* [39, 40]. In endothelial cells, *Ang1* induces *Tie2* phosphorylation [41]; *Ang2* inhibits *Ang1*-induced *Tie2* activation unless endothelial cells undergo prolonged exposure to higher concentrations of *Ang2*. Targeted *Tie1* disruption results in embryonic lethality due to edema, hemorrhage and microvessel rupture [42–44]. *Tie1*-deficient embryos contain hyperactive endothelial cells exhibiting a large number of extensions and filopodia projecting into the vessel lumen and have increased capillary densities, suggesting that *Tie1* may act as a brake during embryonic angiogenesis [44]. Overexpression of *Tie1* mRNA in the bi-maternal placenta may have resulted in their poorly developed vasculature. In the $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg placentae, the *Ang1* transcription level was restored, indicating that appropriate transcription of this gene is highly sensitive to the *Igf2* mRNA expression level.

Differential effects of Igf2 and H19 mRNA expression on angiogenic factor transcription in the fetus and placenta

In addition to placentae, we also carried out quantitative gene

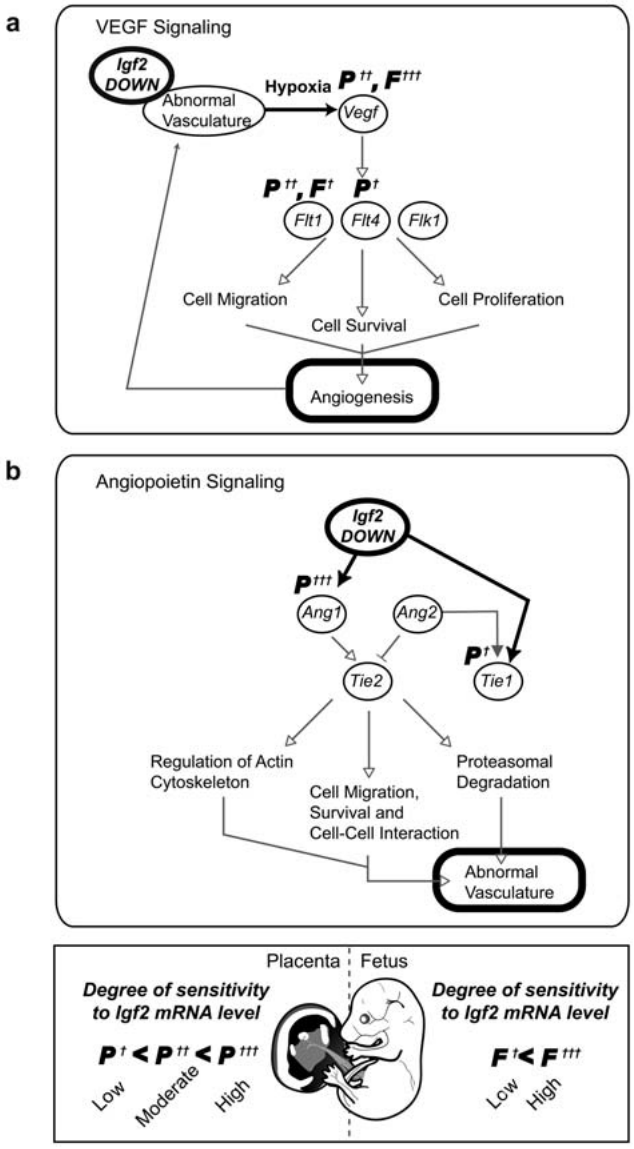

Fig. 5. Summary of the effects of the paternally methylated imprinted genes regulated by the *Igf2-H19* domain on angiogenic factor transcriptions in the mouse placenta and fetus. a: VEGF signaling. b: angiopoietin signaling constructed on the basis of known transcription cascades represented by Ingenuity Pathway Analysis (IPA). Our results were further introduced into these signaling pathways. A detailed description of IPA can be found at www.ingenuity.com. The figure in the bottommost panel represents the differences in the degree of sensitivity that the angiogenic factor mRNA levels had to the *Igf2* transcription level (†, †† and ††† low, moderate and high, respectively). Each dagger mark corresponds directly to the marks shown in Figs. 3 and 4.

expression analysis in bi-maternal fetuses (Fig. 4). In contrast to the results for the placentae, only 2 genes among the 8 genes analyzed were significantly affected in the bi-maternal fetuses. *Flt1* mRNA expression was corrected in the $ng^{H19\Delta3\text{-}KO}$/fg fetus; however, this result was unexpected because *Flt1* transcription in the $ng^{H19\Delta13\text{-}KO}$/fg fetus was not restored. We do not have a clear explanation for this finding. Interestingly, the *Vegf* mRNA expression levels in both the $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg fetuses were generally the same as in the wt fetus, but not in the ng^{wt}/fg fetus. This phenomenon was entirely different from the result obtained by quantitative gene expression analysis for the placenta, indicating an evident difference in the effects on angiogenic factor transcription between the placenta and the fetus (Fig. 5). Our results appear to be consistent with a previous report indicating that placental phenotype depends on the interplay between placental and fetal *Igf2* in the mouse [45]. Previously, the reason for death of $ng^{H19\Delta3\text{-}KO}$/fg conceptuses at E17.5 was unclear, but we hypothesized that defects in the placenta, rather than in the fetus itself, were the main reason for lethality based on a histological assessment [15]. The present study confirms the above hypothesis concerning the differential propagating effects of the *Igf2* mRNA expression level.

To explore the manner in which the *Igf2* mRNA expression level contributes to vasculogenesis/angiogenesis in the bi-maternal placenta and fetus, we performed quantitative gene expression analysis on 8 representative angiogenic factors—*Vegf*, *Flt1*, *Flt4*, *Flk1*, *Ang1*, *Ang2*, *Tie1* and *Tie2*—in 3 types of bi-maternal placentae and fetuses, ng^{wt}/fg, $ng^{H19\Delta3\text{-}KO}$/fg and $ng^{H19\Delta13\text{-}KO}$/fg genotypes. In the bi-maternal conceptuses, we demonstrated that transcription levels of angiogenic factors could be altered in an *Igf2* mRNA expression dose-dependent manner and further found that the propagating effects on the transcription levels of angiogenic factors are different between the placenta and the fetus (Fig. 5). Thus, studying bi-maternal conceptuses provides further insights into the mechanisms by which paternally methylated imprinted genes containing *Igf2* regulate mammalian ontogenesis.

Acknowledgments

We thank Dr S Tilghman, Princeton University, and Dr L Dandolo, Université Paris Descartes, for providing us with the mutant mice. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (No. 20062009 to TK) and Young Scientists (B) (No. 20780201 to MK) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. **Reik W**. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 2007; 447: 425–432.
2. **Edwards CA, Ferguson-Smith AC**. Mechanisms regulating imprinted genes in clusters. *Curr Opin Cell Biol* 2007; 19: 281–289.
3. **Kobayashi H, Suda C, Abe T, Kohara Y, Ikemura T, Sasaki H**. Bisulfite sequencing and dinucleotide content analysis of 15 imprinted mouse differentially methylated regions (DMRs): paternally methylated DMRs contain less CpGs than maternally methylated DMRs. *Cytogenet Genome Res* 2006; 113: 130–137.
4. **Kono T, Obata Y, Yoshimzu T, Nakahara T, Carroll J**. Epigenetic modifications during oocyte growth correlates with extended parthenogenetic development in the mouse. *Nat Genet* 1996; 13: 91–94.
5. **Obata Y, Kaneko-Ishino T, Koide T, Takai Y, Ueda T, Domeki I, Shiroishi T, Ishino F, Kono T**. Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development* 1998; 125: 1553–1560.
6. **Ogawa H, Wu Q, Komiyama J, Obata Y, Kono T**. Disruption of parental-specific expression of imprinted genes in uniparental fetuses. *FEBS Lett* 2006; 580: 5377–5384.

7. **Obata Y, Kono T**. Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *J Biol Chem* 2002; 277: 5285–5289.
8. **Kono T, Obata Y, Wu Q, Niwa K, Ono Y, Yamamoto Y, Park ES, Seo JS, Ogawa H**. Birth of parthenogenetic mice that can develop to adulthood. *Nature* 2004; 428: 860–864.
9. **Bartolomei MS, Zemel S, Tilghman SM**. Parental imprinting of the mouse H19 gene. *Nature* 1991; 351: 153–155.
10. **Gabory A, Ripoche MA, Yoshimizu T, Dandolo L**. The H19 gene: regulation and function of a non-coding RNA. *Cytogenet Genome Res* 2006; 113: 188–193.
11. **DeChiara TM, Robertson EJ, Efstratiadis A**. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 1991; 64: 849–859.
12. **Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM**. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 1995; 375: 34–39.
13. **Ripoche MA, Kress C, Poirier F, Dandolo L**. Deletion of the H19 transcription unit reveals the existence of a putative imprinting control element. *Genes Dev* 1997; 11: 1596–1604.
14. **Wang ZQ, Fung MR, Barlow DP, Wagner EF**. Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* 1994; 372: 464–467.
15. **Kono T, Sotomaru Y, Katsuzawa Y, Dandolo L**. Mouse parthenogenetic embryos with monoallelic H19 expression can develop to day 17.5 of gestation. *Dev Biol* 2002; 243: 294–300.
16. **Kawahara M, Wu Q, Yaguchi Y, Ferguson-Smith AC, Kono T**. Complementary roles of genes regulated by two paternally methylated imprinted regions on chromosomes 7 and 12 in mouse placentation. *Hum Mol Genet* 2006; 15: 2869–2879.
17. **Sun FL, Dean WL, Kelsey G, Allen ND, Reik W**. Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome. *Nature* 1997; 389: 809–815.
18. **Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A**. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev* 1997; 11: 3128–3142.
19. **Chao W, D'Amore PA**. IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev* 2008; 19: 111–120.
20. **Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E**. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem* 2002; 277: 27975–27981.
21. **Yoshimizu T, Miroglio A, Ripoche MA, Gabory A, Vernucci M, Riccio A, Colnot S, Godard C, Terris B, Jammes H, Dandolo L**. The H19 locus acts *in vivo* as a tumor suppressor. *Proc Natl Acad Sci USA* 2008; 105: 12417–12422.
22. **Ainscough JF, Koide T, Tada M, Barton S, Surani MA**. Imprinting of Igf2 and H19 from a 130 kb YAC transgene. *Development* 1997; 124: 3621–3632.
23. **Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N**. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989; 246: 1306–1309.
24. **Breier G**. Angiogenesis in embryonic development—a review. *Placenta* 2000; 21 (Suppl A): S11–15.
25. **Ferrara N, Davis-Smyth T**. The biology of vascular endothelial growth factor. *Endocr Rev* 1997; 18: 4–25.
26. **Thomas M, Augustin HG**. The role of the angiopoietins in vascular morphogenesis. *Angiogenesis* 2009; 12: 125–137.
27. **Kawahara M, Obata Y, Sotomaru Y, Shimozawa N, Bao S, Tsukadaira T, Fukuda A, Kono T**. Protocol for the production of viable bimaternal mouse embryos. *Nature Protocols* 2008; 3: 197–209.
28. **Whittingham DG**. Culture of mouse ova. *J Reprod Fertil Suppl* 1971; 14: 7–21.
29. **Kawahara M, Wu Q, Ferguson-Smith AC, Kono T**. Appropriate expression of imprinted genes on mouse chromosome 12 extends development of bi-maternal embryos to term. *FEBS Lett* 2007; 581: 5178–5184.
30. **Kawahara M, Wu Q, Takahashi N, Morita S, Yamada K, Ito M, Ferguson-Smith AC, Kono T**. High-frequency generation of viable mice from engineered bi-maternal embryos. *Nat Biotechnol* 2007; 25: 1045–1050.
31. **Geva E, Ginzinger DG, Zaloudek CJ, Moore DH, Byrne A, Jaffe RB**. Human placental vascular development: vasculogenic and angiogenic (branching and nonbranching) transformation is regulated by vascular endothelial growth factor-A, angiopoietin-1, and angiopoietin-2. *J Clin Endocrinol Metab* 2002; 87: 4213–4224.
32. **Reik W, Constancia M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, Tycko B, Sibley C**. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J Physiol* 2003; 547: 35–44.
33. **Rahnama F, Shafiei F, Gluckman PD, Mitchell MD, Lobie PE**. Epigenetic regulation of human trophoblastic cell migration and invasion. *Endocrinology* 2006; 147: 5275–5283.
34. **Mayer W, Hemberger M, Frank HG, Grummer R, Winterhager E, Kaufmann P, Fundele R**. Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. *Dev Dyn* 2000; 217: 1–10.
35. **Gerber HP, Condorelli F, Park J, Ferrara N**. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem* 1997; 272: 23659–23667.
36. **Nagamatsu T, Fujii T, Kusumi M, Zou L, Yamashita T, Osuga Y, Momoeda M, Kozuma S, Taketani Y**. Cytotrophoblasts up-regulate soluble fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia. *Endocrinology* 2004; 145: 4838–4845.
37. **Ferrara N**. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; 25: 581–611.
38. **Simiantonaki N, Jayasinghe C, Michel-Schmidt R, Peters K, Hermanns MI, Kirkpatrick CJ**. Hypoxia-induced epithelial VEGF-C/VEGFR-3 upregulation in carcinoma cell lines. *Int J Oncol* 2008; 32: 585–592.
39. **Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J**. Vascular-specific growth factors and blood vessel formation. *Nature* 2000; 407: 242–248.
40. **Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L, Dewhirst MW, Sankar S**. Functional significance of Tie2 signaling in the adult vasculature. *Recent Prog Horm Res* 2004; 59: 51–71.
41. **Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD**. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* 1997; 277: 55–60.
42. **Puri MC, Rossant J, Alitalo K, Bernstein A, Partanen J**. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J* 1995; 14: 5884–5891.
43. **Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y**. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 1995; 376: 70–74.
44. **Patan S**. TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. *Microvasc Res* 1998; 56: 1–21.
45. **Coan PM, Fowden AL, Constancia M, Ferguson-Smith AC, Burton GJ, Sibley CP**. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. *J Physiol* 2008; 586: 5023–5032.