Increased expression of vascular endothelial growth factor in placentas of p57^{Kip2} null embryos

Toshiki Matsuura[a,1,*], Katsuhiko Takahashi[b,1], Keiko Nakayama[c], Takao Kobayashi[a], Nam-Ho Choi-Miura[b], Motowo Tomita[b], Naohiro Kanayama[a]

[a] *Department of Obstetrics and Gynecology, Hamamatsu University School of Medicine 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[b] *Department of Physiological Chemistry, School of Pharmaceutical Sciences, Showa University 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan*
[c] *Laboratory of Embryonic and Genetic Engineering, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, Fukuoka 812-8582, Japan*

Received 25 September 2002; revised 29 October 2002; accepted 29 October 2002

First published online 14 November 2002

Edited by Ned Mantei

Abstract Placentas of mice lacking p57^{Kip2} expression have trophoblastic hyperplasia. To elucidate the mechanism underlying this phenomenon, we studied expression of two angiogenic factors, vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF). Immunohistochemical analysis with anti-VEGF antibodies indicated that VEGF expression was stronger and more clearly detectable in placentas of p57^{Kip2} null embryos compared to wild-type placentas. PlGF showed no significant differences between placentas of p57^{Kip2} null and wild-type embryos. In quantitative analysis, placentas of p57^{Kip2} null embryos showed higher VEGF messenger (m)RNA and protein levels than did wild-type placentas. PlGF mRNA and protein levels were not significantly different. These findings suggest that VEGF is involved in the hyperplasia that occurs in placentas of p57^{Kip2} null embryos.

Key words: p57^{Kip2}; Genomic imprinting; Placentation; Preeclampsia; VEGF

1. Introduction

Proper development of the placenta is dependent on the formation of trophoblasts. In humans, placental abnormalities with trophoblastic dysplasia are observed in several pregnancy-related diseases including hydatidiform mole, choriocarcinoma, pregnancy-induced diabetes and preeclampsia. However, there are few animal models of placentomegaly and trophoblast proliferation.

*Corresponding author. Fax: (81)-53-435 2308.
E-mail addresses: matsuura@hama-med.ac.jp (T. Matsuura), takahask@pharm.showa-u.ac.jp (K. Takahashi), nakayak2@bioreg.kyushu-u.ac.jp (K. Nakayama), tkoba@hama-med.ac.jp (T. Kobayashi), nammiura@pharm.showa-u.ac.jp (N.-H. Choi-Miura), tomitam@pharm.showa-u.ac.jp (M. Tomita), kanayama@hama-med.ac.jp (N. Kanayama).

[1] These authors contributed equally to this work.

Abbreviations: VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA; PCR, polymerase chain reaction; RT, reverse transcription

In mammals, imprinted genes are expressed from one of the parental chromosomes and are crucial for placentation [1]. Recent studies have revealed that imprinted genes are closely related to trophoblastic disease [2]. For example, genomic imprinting is important in the generation of the hydatidiform mole, which is characterized by absence of the maternal genome and grossly swollen villi [3].

The cell cycle of trophoblastic cells is regulated by cyclins as well as other factors. The cyclin-dependent kinase (CDK) inhibitor p57^{Kip2} has the ability to bind to a variety of cyclin–CDK complexes and to inhibit their kinase activities in vitro. The p57^{Kip2} gene is a paternally imprinted gene that is transcriptionally repressed and methylated, and is therefore expressed predominantly from the maternal allele. The p57^{Kip2} gene is located within a cluster of imprinted genes in both humans (chromosome 11p15.5) and mice (distal chromosome 7). In humans, loss of the maternally derived 11p15.5 region is implicated in sporadic tumors and in Beckwith–Wiedemann syndrome (BWS), which is characterized by congenital malformations and organomegaly and is associated with an increased risk for the development of childhood neoplasms. Abnormal expression of imprinted genes is implicated in the pathogenesis of certain pediatric tumors. p57^{Kip2} mutations have been found only rarely in association with BWS, but a decrease in p57^{Kip2} expression levels has been detected in several kinds of tumors [4,5].

Mice deficient in the p57^{Kip2} gene show defective endochondral bone formation. Most of these mice die shortly after birth, as a result of severe cleft palate. Zhang et al. reported that p57^{Kip2} knockouts display organomegaly and abdominal wall defects, two of the hallmarks of BWS, and they found no p57^{Kip2} deficient mice that survived beyond the neonatal period [6]. To the contrary, Yan et al. and Takahashi et al. reported that p57^{Kip2} deficient mice displayed no features of BWS, and they observed a 10% survival rate [7,8]. The reports of surviving mutant mice did not indicate it p57^{Kip2} deficient mice had tumorous tissues.

Changes in p57^{Kip2} expression have recently been associated with abnormal trophoblastic proliferation [9]. That is, p57^{Kip2} expression has been shown to be reduced markedly in women with malignant trophoblastic neoplasms that cause spontaneous abortion and preterm delivery. Furthermore, placentas of p57^{Kip2} null embryos have hyperplasia involving both labyrinthine trophoblasts and spongiotrophoblasts [10]. Placentation

0014-5793/02/$22.00 © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(02)03681-5

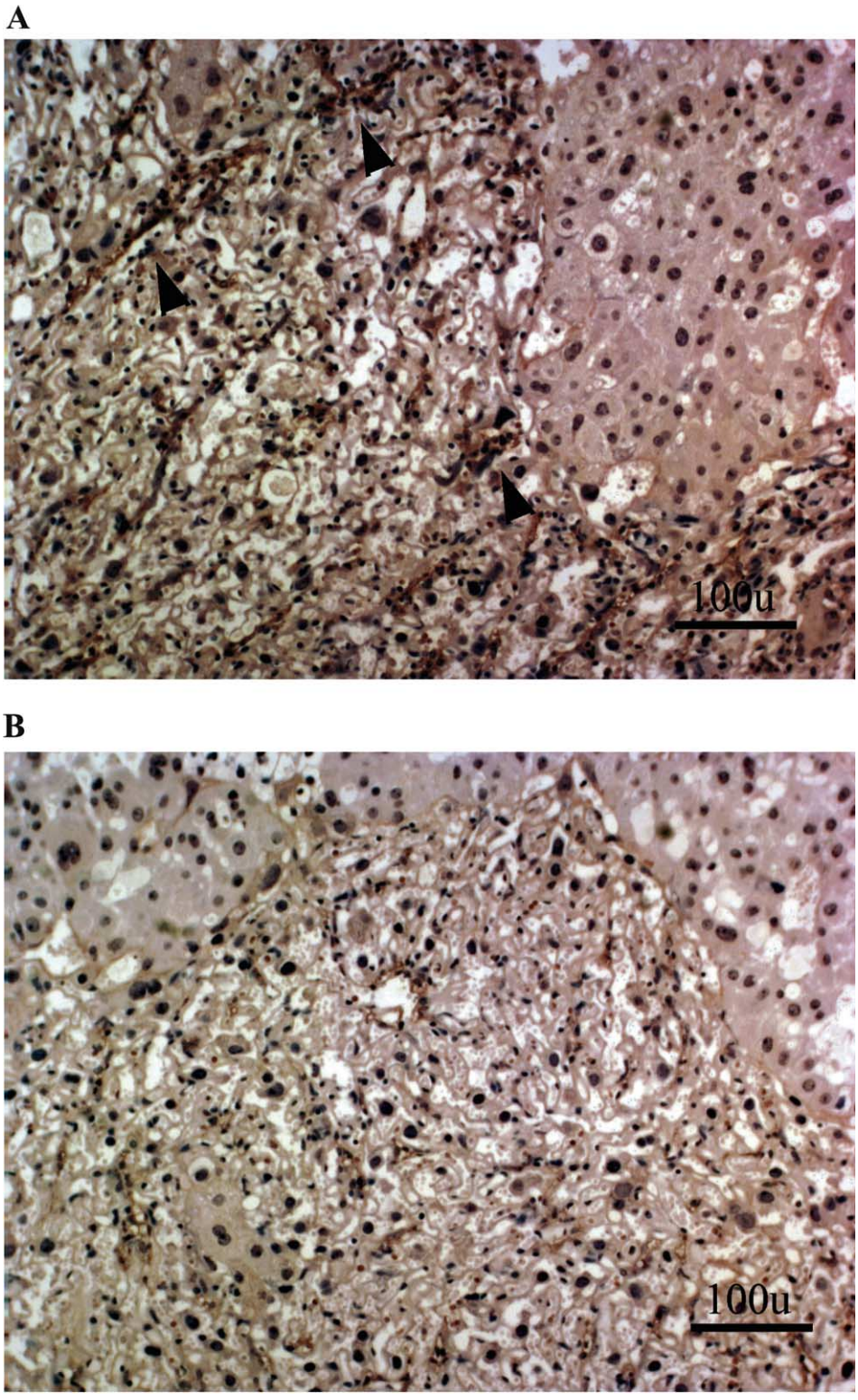

Fig. 1. Histologic findings of placentas from $p57^{Kip2}$ null embryos and wild-type embryos. Detection of VEGF by immunoperoxidase staining in placentas of $p57^{Kip2}$ null embryo (17.5 d.p.c.) (A) and wild-type embryo (17.5 d.p.c.) (B). In the labyrinthine trophoblast layer, VEGF-positive cells are more abundant in placentas of $p57^{Kip2}$ null embryo in comparison to wild-type placentas, as indicated by the arrow.

is thought to be controlled by cytokines, growth factors, and hypothalamic hormones that are secreted by endocrine organs including the placenta. Vascular endothelial growth factor (VEGF) and placenta growth factor (PlGF) are the best characterized angiogenic factors with respect to placentation [11,12].

The purpose of the present study was to clarify the relationship between VEGF, PlGF and trophoblast proliferation in

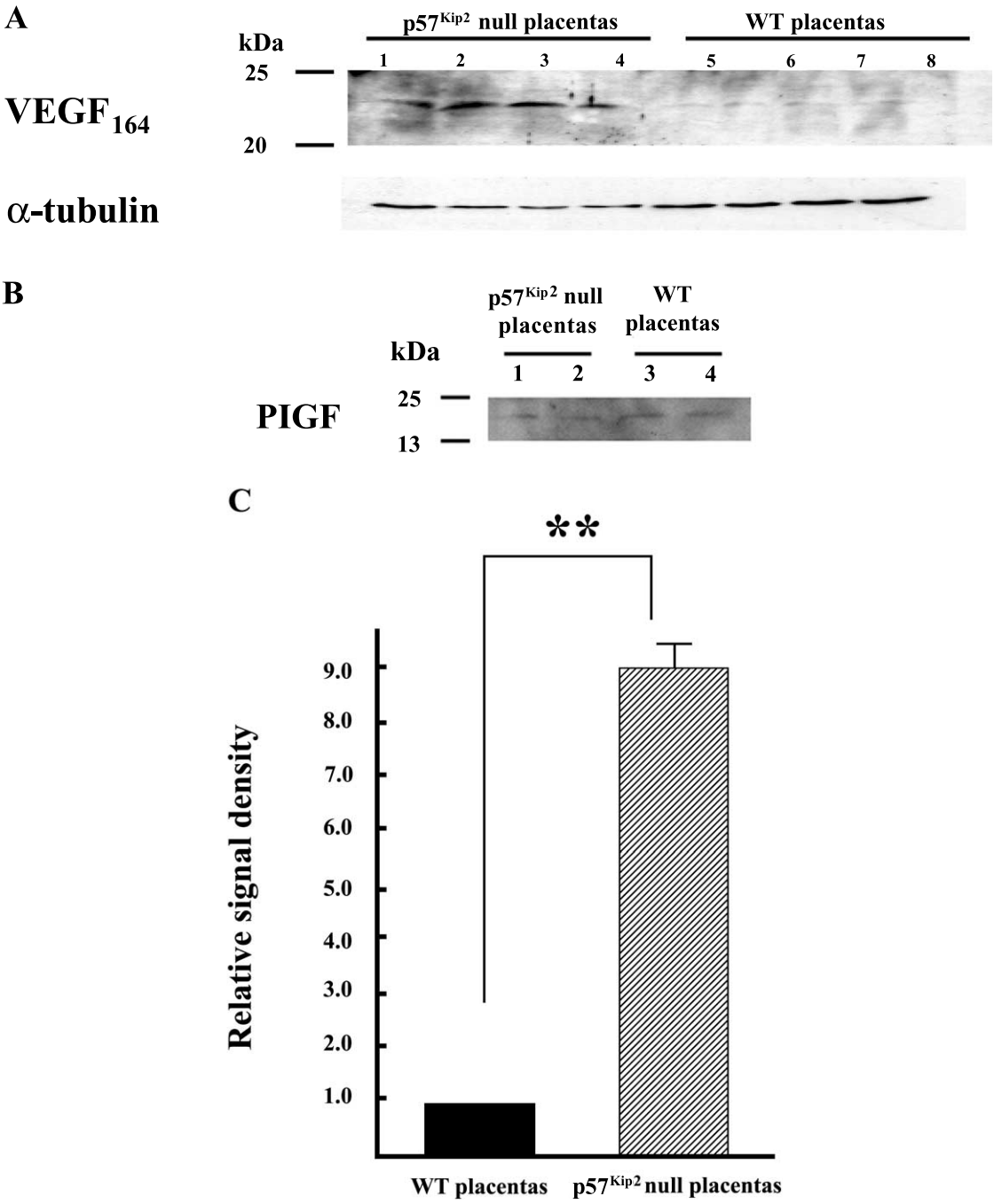

Fig. 2. Immunoblot analysis of VEGF and PlGF proteins in placentas of $p57^{Kip2}$ null embryos and wild-type embryos. A: $VEGF_{164}$; lanes 1–4 show placentas of $p57^{Kip2}$ null embryos. Lanes 5–8 show wild-type placentas. Alpha-tubulin expression from the same samples served as an internal control to verify equal protein loading. B: PlGF; lanes 1 and 2 show placentas of $p57^{Kip2}$ null embryos. Lanes 3 and 4 show wild-type placentas. C: Densitometric analysis of $VEGF_{164}$ protein. Relative expression levels were obtained, in each sample, by normalization of absolute $VEGF_{164}$ of the specific target to that of alpha-tubulin signal. Error bars indicate standard deviation. $^{**}P < 0.01$ compared with wild-type embryos.

placentas of $p57^{Kip2}$ null embryos. Both VEGF and PlGF stimulate migration and proliferation of endothelial cells. In embryos, alternative splicing yields at least three different VEGF isoforms, $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ [13]. VEGF has been implicated in the process of neovascularization during organogenesis and regeneration, as well as in the process of blood vessel proliferation during tumor formation [14]. VEGF increase also causes increased vascular permeability [15]. Many investigators have suggested that VEGF plays an important role in villous angiogenesis and trophoblast differentiation [16–18]. However, it is unknown whether placental levels of VEGF are increased in patients with pregnancy-induced disease. In the present study we investigated the expression of VEGF and PlGF protein and messenger (m)RNA levels in placentas of $p57^{Kip2}$ null embryos.

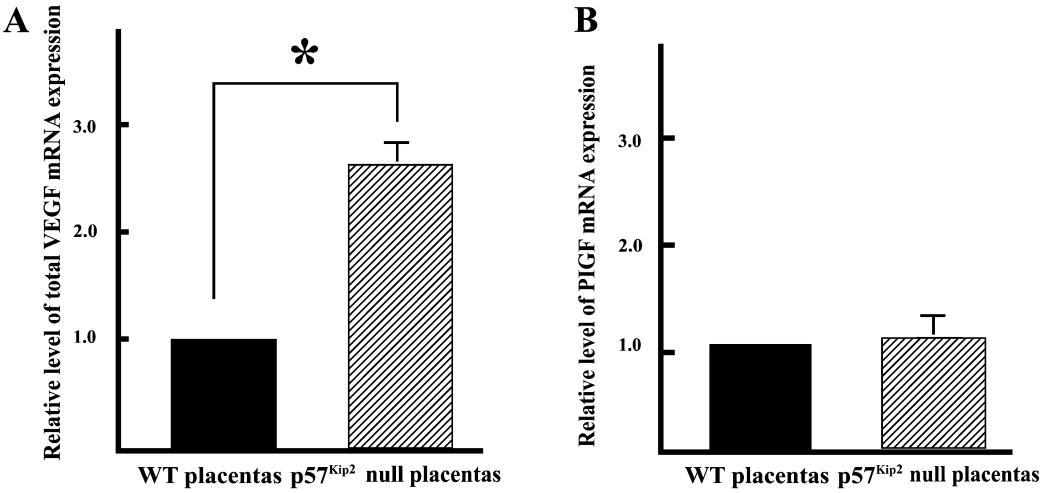

Fig. 3. Quantitative analysis of total VEGF and PlGF mRNAs in placentas of $p57^{Kip2}$ null embryos and wild-type embryos. Total VEGF and PlGF mRNA levels were determined by real-time RT-PCR. GAPDH was used to normalize these data. A: Relative increase of total VEGF mRNA; $*P < 0.05$ compared with wild-type embryos. B: Relative increase of PlGF mRNA; error bars indicate standard deviation. The amounts of standard samples were plotted versus threshold cycle in duplicate (data not shown).

2. Materials and methods

2.1. Mice

The mice used in this study carry a targeted mutation in the $p57^{Kip2}$ locus and were provided by Nippon Roche Research Center. The materials derived from $p57^{Kip2}$ null embryos were genotyped by polymerase chain reaction (PCR) [8].

2.2. Immunohistological analysis

All placentas were placed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and then in PBS with 10% sucrose until they were processed and embedded in paraffin. The sections were cut (4 μm thick). After being dried overnight at room temperature, sections were deparaffinized with xylene. Sections were then rehydrated through graded ethanols.

Specimens were treated with 0.3% H_2O_2 for 10 min to block endogenous peroxidase activity, and preimmune goat serum was used to block non-specific binding sites. Sections were then incubated overnight at 4°C with anti-VEGF antibody (Neo markers, Fremont, CA, USA). After three washes with PBS, sections were incubated with biotinylated secondary antibody followed after washing by streptavidin biotin peroxidase. Sections were counterstained with hematoxylin.

2.3. Immunoblot analysis

Levels of VEGF and PlGF proteins were assessed by Western blotting. Total lysates of placentas were prepared with Tween 20 lysis buffer as described previously [8]. Protein concentrations were determined with the Bradford method (protein assay; Bio-Rad, Hercules, CA, USA). Total lysates (50 μg/lane) were separated by electrophoresis on 15% polyacrylamide gels and transferred to Immobilon transfer membranes (Millipore, Bedford, MA, USA). We used polyclonal rabbit anti-mouse VEGF and goat anti-mouse PlGF antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies. The secondary antibodies were peroxidase-conjugated sheep anti-rabbit antibody (for VEGF, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-goat antibody (for PlGF, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The signal was visualized with enhanced chemiluminescence (ECL) detection system according to the manufacturer's instructions. Relative intensities of VEGF protein levels were analyzed densitometrically (ATTO Corporation, Tokyo, Japan) and compared with Image Saver software (ATTO Corporation, Tokyo, Japan). Protein levels were normalized to those of alpha-tubulin.

All experiments were repeated in triplicate, and mean intensities ± standard deviations were calculated.

2.4. Quantitative real-time reverse transcription (RT)-PCR

RT reaction of 2 μg total RNA was carried out using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA). Samples were incubated at 42°C for 60 min, at 95°C for 2 min, and finally cooled on ice.

Real-time PCR from VEGF and PlGF cDNA templates was performed with the use of SYBR Green PCR buffer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) containing 0.4 μM each primer, 0.6 U AmpliTaq Gold DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA), and dNTP blend. Each reaction contained 100 ng of cDNA as templates. The reaction were run in the well after optimizing the conditions for multiplexing, as described previously [19]. The reactions were performed in a final volumes of 50 μl. The each primers were follows; VEGF (a) sense and VEGF (a) anti-sense (accession number NM_009505 VEGF (a) sense 5′-GCA CAC AGG ACG GCT TGA AGA T-3′ position 168 and VEGF (a) anti-sense 5′-CCC ACG ACA GAA GAG CAG A-3′ position 297, product length 151 bp) and PlGF sense and PlGF anti-sense (accession number NM_008827, forward primer 5′-CCC ACA CCC AGC TCA CGT ATT TA-3′ and reverse primer 5′-TCC CCT CTA CAT GCC TTC AAT GC-3′). The thermal cycling conditions of the ABI PRISM 5700 Sequence Detection instrument (Applied Biosystems, Foster City, CA, USA) were used with 40 cycles of 15 s at 95°C alternating with 1 min 60°C. Samples were analyzed in triplicate. As control reaction, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included in each run and the specificity of the amplification reaction was checked by melting curve analysis. PCR data were obtained by considering the log-linear portion of the fluorescence curve. Analysis of these data was done as described previously [20].

2.5. RT-PCR

RT reactions were described in Section 2.4. The nucleotide sequences of the primers were VEGF (forward primer 5′-ACA TCT TCA AGC CGT CCT GTG TGC-3′ and reverse primer 5′-AAA TGG CGA ATC CAG TCC CAC GAG-3′) [21] and PlGF (primers were those described in Section 2.4). Primers spanning the VEGF gene amplified the three reported mRNA variants, $VEGF_{120}$, $VEGF_{164}$, $VEGF_{188}$, as the expected 431, 563, and 635 bp products, respectively. Primers spanning the PlGF gene amplified 364 bp products. Reactions were carried out in a DNA thermal cycler 480 (Takara, Tokyo, Japan). Taq DNA polymerase was used for hot-start (94°C for 10 min) prior to the amplification cycles, and a terminal elongation step (72°C for 10 min) following the amplification cycles.

Thirty cycles were used for the PCR amplification. The cycling temperatures and timings for VEGF gene were: 94°C for 1 min, 62°C for 1.5 min, and 72°C for 1 min. The cycling temperatures and timings for PlGF gene were: 94°C for 1 min, 61°C for 1.5 min, and 72°C for 1 min.

PCR products were resolved on 2.0% agarose/TAE gels containing

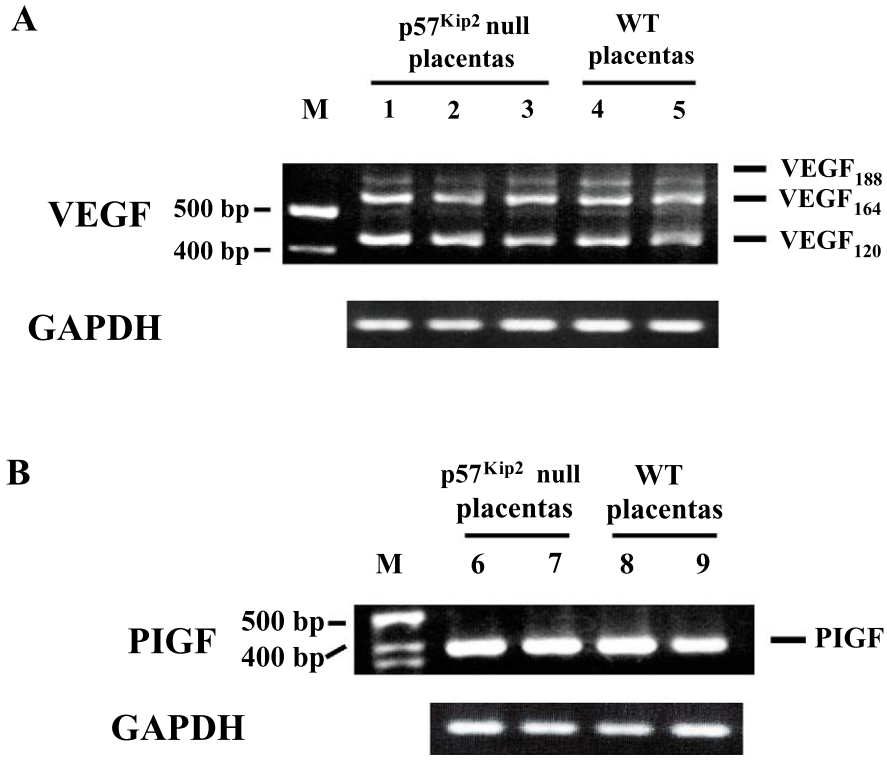

Fig. 4. Expression of VEGF isoforms (A) and PlGF mRNA (B) mRNA in placentas of $p57^{Kip2}$ null embryos and wild-type embryos. Reverse-transcribed RNA was amplified by PCR with primers derived from exons shared by $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ mRNA species, which thus amplify all VEGF and PlGF isoforms. Products of 431, 563, and 635 bp correspond to the isoforms indicated at the right. The PlGF product is 364 bp. The corresponding amplification of GAPDH mRNA from the same RT reaction is depicted; GAPDH product comprises 450 bp. Lanes 1, 2, 3, 6 and 7 show placentas of $p57^{Kip2}$ null embryos. Lanes 4, 5, 8 and 9 show placentas of wild-type embryos. M = size marker.

3 μg/ml ethidium bromide and visualized under UV light. VEGF and PlGF levels were normalized to those of housekeeping gene GAPDH.

2.6. Statistical analysis

All data were analyzed using Student's *t*-test. Differences were considered to be statistically significant if $P < 0.05$.

3. Results

3.1. Increase of $VEGF_{164}$ protein levels in placentas of $p57^{Kip2}$ null embryos

Immunohistochemical analysis with anti-VEGF specific antibody showed strong positive staining for VEGF in the labyrinthine layers of placentas of $p57^{Kip2}$ null embryos, as compared with those of wild-type embryos. (Fig. 1A,B) It had been reported that placentas of $p57^{Kip2}$ null embryos displayed trophoblastic hyperplasia.

Immunoblot analysis was used access the VEGF protein levels. VEGF isoforms were identified by apparent molecular weight. $VEGF_{164}$ protein levels in placentas of $p57^{Kip2}$ null embryos were higher than those of wild-type embryos (Fig. 2A). $VEGF_{120}$, $VEGF_{164}$ and $VEGF_{188}$ protein levels were compared in placentas of $p57^{Kip2}$ null and wild-type embryos by using densitometric analysis. $VEGF_{164}$ protein levels were approximately nine-fold higher in placentas of $p57^{Kip2}$ null embryos than in those of wild-type embryos (Fig. 2C). However $VEGF_{120}$ and $VEGF_{188}$ were detected, these two protein levels did not differ significantly (data not shown). Levels of PlGF protein did not differ significantly between placentas of $p57^{Kip2}$ null embryos and wild-type embryos (Fig. 2B).

3.2. Increase of total VEGF mRNA levels in placentas of $p57^{Kip2}$ null embryos

We measured total VEGF mRNA levels to investigate whether the increase of VEGF protein levels was caused by an increase of VEGF mRNA levels.

Real-time RT-PCR quantification of total VEGF and PlGF mRNA levels in placentas of $p57^{Kip2}$ null embryos and wild-type embryos is shown in Fig. 3. These values were normalized to GAPDH. Total VEGF mRNA levels in placentas of $p57^{Kip2}$ null embryos were approximately 2.5-fold higher than in placentas of wild-type embryos (Fig. 3A). PlGF mRNA levels were not significantly different between the groups (Fig. 3B). Other PCR primer sets were used to distinguish each VEGF isoform. RT-PCR showed that transcripts for $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ showed similar relative levels in placentas of both $p57^{Kip2}$ null embryos and wild-type embryos (Fig. 4A). Note that amplification efficiencies may vary for the different products, so that relative amounts of the various isoforms within one sample cannot be determined. Expression of PlGF again did not differ significantly between placenta of $p57^{Kip2}$ null embryos and wild-type placentas (Fig. 4B).

4. Discussion

The purpose of this research was to clarify the relationship between (i) VEGF and PlGF expression levels and (ii) trophoblastic proliferation in placentas of $p57^{Kip2}$ null embryos. We previously reported that $p57^{Kip2}$ contributes to proper devel-

opment of labyrinthine trophoblasts and spongiotrophoblasts through pathways not associated with regulation of CDK activity, as assessed by analysis of $p57^{Kip2}$ null mice [10]. We found that pregnant $p57^{Kip2}$ heterozygous female mice that gestated embryos lacking $p57^{Kip2}$ expression have hypertension, proteinuria, thrombocytopenia, decreased antithrombin III activity, and increased endothelin levels during late pregnancy [22]. In this study, we found using immunohistochemical analysis that VEGF is expressed at high levels in the labyrinthine layers of $p57^{Kip2}$ null embryos. Furthermore, our findings indicate that levels of total VEGF mRNA and $VEGF_{164}$ protein levels are higher in placentas of $p57^{Kip2}$ null embryos than in those of wild-type embryos. We found that placentas of $p57^{Kip2}$ null embryos express the same complement of VEGF isoforms by using RT-PCR. This suggests that $p57^{Kip2}$ does not markedly affect VEGF splicing, although an increase in specifically the $VEGF_{164}$ isoform might have been expected based on the Western blot analysis. We have at present no explanation for this discrepancy. $VEGF_{164}$ plays an important role in placentation [23]. PlGF mRNA and protein levels were not significantly different between placentas of $p57^{Kip2}$ null embryos and wild-type embryos. These results reveal that there is a close association between VEGF levels and placentation.

Among VEGF isoforms, $VEGF_{164}$ protein levels were significantly increased in placentas of $p57^{Kip2}$ null embryos. $VEGF_{164}$ shows more potent induction of tumorigenesis than do the other VEGF isoforms in human [24]. Our data sheds some light on the proliferation of trophoblasts in preeclampsia, which is induced placental hyperplasia.

The loss of $p57^{Kip2}$ expression is involved in abnormal androgenic proliferation of trophoblasts [9]. $p57^{Kip2}$ may also have marked effects on several stages of placental development and preeclampsia. It has been assumed that preeclampsia is due to deficient trophoblast invasion of the placental bed spiral arteries, resulting in poor perfusion of the fetoplacental unit and placenta, which leads to secretion of vasoactive substances into the maternal circulation. Because VEGF is expressed in the placenta and VEGF production can be induced by hypoxia, changes in levels of VEGF may contribute to the etiology of preeclampsia.

However, it is unclear whether placental VEGF levels are associated with serum VEGF levels in preeclampsia [25,26]. Cooper, et al. [27], reported that expression of VEGF mRNA is reduced significantly in placentas of women with preeclampsia in comparison to placentas of normal pregnant women. In contrast, Brockelsby, et al. [28] reported that placentally derived VEGF appears to be responsible for the increased circulating levels of VEGF in preeclampsia.

In general, the birth of a healthy infant at term is dependent upon normal placental development. Conversely, abnormal placentation is responsible for a wide range of pregnancy-related complications, including miscarriage, preeclampsia, intrauterine growth retardation, and placental abruption. In the future, study of placentas of $p57^{Kip2}$ null embryos may clarify the mechanisms involved in early and late placental dysfunction and trophoblast proliferation. From this standpoint, we think that placentas of $p57^{Kip2}$ null embryos provide a new animal model of trophoblastic hyperplasia with VEGF increase and a new means of studying the mechanisms of defective placentation.

Acknowledgements: We are grateful to K. Nakayama, H. Amano, M. Yoda, K. Saito and Y. Sano for providing their expert knowledge, and A. Shinoda, T. Suzuki, and T. Tomuro for excellent technical assistance. We wish to thank A. Rajkovic for his suggestions and K.H. Burns for critical review of manuscript.

References

[1] Reik, W. and Walter, J. Genomic imprinting: parental influence on the genome, (2001) Nat. Rev. Genet. 2, 21–32.
[2] Castrillon, D.H., Sun, D., Weremowicz, S., Fisher, R.A., Crum, C.P. and Genest, D.R. (2001) Am. J. Surg. Pathol. 25, 1225–1230.
[3] Wake, N., Arima, T. and Matsuda, T. (1998) Int. J. Gynaecol. Obstet. 60, S1–S8.
[4] Gaston, V., Le Bouc, Y., Soupre, V., Vazquez, M.P. and Gicquel, C. (2000) Horm. Res. 54, 1–5.
[5] Lee, M.H. and Yang, H.Y. (2001) Cell Mol. Life Sci. 58, 1907–1922.
[6] Zhang, P., Liegeois, N.J., Wong, C., Finegold, M., Hou, H., Thompson, J.C., Silverman, A., Harper, J.W., DePinho, R.A. and Elledge, S.J. (1997) Nature 387, 151–158.
[7] Yan, Y., Frisen, J., Lee, M.H., Massague, J. and Barbacid, M. (1997) Genes. Dev. 11, 973–983.
[8] Takahashi, K., Nakayama, K. and Nakayama, K. (2000) J. Biochem. (Tokyo) 127, 73–83.
[9] Chilosi, M., Piazzola, E., Lestani, M., Benedetti, A., Guasparri, I., Granchelli, G., Aldovini, D., Leonardi, E., Pizzolo, G., Doglioni, C., Menestrina, F. and Mariuzzi, G.M. (1998) Lab. Invest. 78, 269–276.
[10] Takahashi, K., Kobayashi, T. and Kanayama, N. (2000) Mol. Hum. Reprod. 6, 1019–1025.
[11] Lash, G.E., Cartwright, J.E., Whitley, G.S., Trew, A.J. and Baker, P.N. (1999) Placenta 20, 661–667.
[12] Ghosh, D., Sharkey, A.M., Charnock-Jones, D.S., Dhawan, L., Dhara, S., Smith, S.K. and Sengupta, J. (2000) Mol. Hum. Reprod. 6, 935–941.
[13] Shima, D.T., Kuroki, M., Deutsch, U., Ng, Y.S., Adamis, A.P. and D'Amore, P.A. (1996) J. Biol. Chem. 271, 3877–3883.
[14] Ferrara, N., Houck, K., Jakeman, L. and Leung, D.W. (1992) Endocr. Rev. 13, 18–32.
[15] Bates, D.O., Lodwick, D. and Williams, B. (1999) Microcirculation 6, 83–96.
[16] Reuvekamp, A., Velsing-Aarts, F.V., Poulina, I.E., Capello, J.J. and Duits, A.J. (1999) Br. J. Obstet. Gynaecol. 106, 1019–1022.
[17] Hefler, L., Obermair, A., Husslein, P., Kainz, C. and Tempfer, C. (2000) Acta Obstet. Gynecol. Scand. 79, 77–78.
[18] Hunter, A., Aitkenhead, M., Caldwell, C., McCracken, G., Wilson, D. and McClure, N. (2000) Hypertension 36, 965–969.
[19] Simpson, D.A., Feeney, S., Boyle, C. and Stitt, A.W. (2000) Mol. Vis. 6, 178–183.
[20] Kubo, A., Nishitani, Y., Minamino, N., Kikumoto, K., Kurioka, H., Nishino, T., Iwano, M., Shiiki, H., Kangawa, K. and Dohi, K. (2000) Nephron 85, 201–206.
[21] Hovey, R.C., Goldhar, A.S., Baffi, J. and Vonderhaar, B.K. (2001) Mol. Endocrinol. 15, 819–831.
[22] Kanayama, N., Takahashi, K., Matsuura, T., Sugimura, M., Kobayashi, T., Moniwa, N., Nakayama, K. and Tomita, M. Mol. Hum. Rep., in press.
[23] Wulff, C., Wilson, H., Dickson, S.E., Wiegand, S.J. and Fraser, H.M. (2002) Biol. Reprod. 66, 802–812.
[24] Grunstein, J., Masbad, J.J., Hickey, R., Giordano, F. and Johnson, R.S. (2000) Mol. Cell. Biol. 20, 7282–7291.
[25] Brockelsby, J.C., Anthony, F.W., Johnson, I.R. and Baker, P.N. (2000) Am. J. Obstet. Gynecol. 182, 176–183.
[26] Jelkmann, W. (2001) Clin. Chem. 47, 617–623.
[27] Cooper, J.C., Sharkey, A.M., Charnock-Jones, D.S., Palmer, C.R. and Smith, S.K. (1996) Br. J. Obstet. Gynaecol. 103, 1191–1196.
[28] Brockelsby, J., Hayman, R., Ahmed, A., Warren, A., Johnson, I. and Baker, P. (1999) Lab. Invest. 79, 1101–1111.