Histol Histopathol (2012) 27: 59-68

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Cell cycle inhibitor p57 expression in normal and diabetic rat placentas during some stages of pregnancy

Nuray Acar, Emin Turkay Korgun and Ismail Ustunel

Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, Antalya, Turkey

Summary. Placentomegaly, an abnormal increase in the size of the placenta, is commonly seen in human diabetic pregnancies and diabetic animal experimental models. Proper placental development depends on the proliferation and differentiation of trophoblasts. However, our knowledge about the mitotic regulators that play key roles in synchronizing these events is limited. p57 is a cyclin-dependent kinase (CDK) inhibitor acting in the G1/S transition of the cell cycle. There is no data regarding p57 expression in either rat or human diabetic placentas. The purpose of this study was to investigate p57 expression in control and diabetic rat placentas at different stages of pregnancy. Diabetes was induced by streptozotocin on the first day of pregnancy, and placentas were taken on days 11, 13, 17, and 21 of pregnancy. Our results showed that on day 11, p57 immunostaining intensity was stronger in control group placentas compared to the diabetic group. On day 13, p57 immunostaining intensity increased in both groups, but increased more in the diabetic group. On day 17, p57 immunostaining intensity decreased in both the control and diabetic groups compared to day 13, yet the intensity remained higher in control placentas compared to diabetic placentas. On day 21 of pregnancy, p57 immunostaining intensity increased in the control group and it decreased from the day 17 level in the diabetic group. Western blot results showed consistency with immunohistochemistry results. Our study shows different expression patterns of p57 between control and diabetic rat placentas, which indicate p57 may play a role in abnormal placental formation resulting in placentomegaly arising from diabetes.

Key words: Diabetes, Placenta, Rat, p57

Offprint requests to: Ismail Ustunel, Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, 07070 Antalya, Turkey. e-mail: iustunel@akdeniz.edu.tr

Introduction

The birth of a healthy infant at term is dependent upon normal placental development. The placenta is the first organ to form during mammalian embryogenesis. Problems in its formation and function underlie many aspects of pregnancy complications (Matsuura et al., 2002; Kim et al., 2005).

Several important aspects of human diabetic pregnancies, such as the increases in the rates of early embryo loss, spontaneous abortions, malformations, fetoplacental impairments, and offspring's diseases in later life, can be studied using the appropriate animal models. Models of diabetes have been widely used to address early embryo developmental defects, placental abnormalities, fetal maldevelopment, and intrauterine transmission of metabolic diseases. Since there is no animal model equal to the human situation, rats have been mostly studied in chemically-induced type 1 diabetes and pregnancy experimental models. The administration of drugs like streptozotocin (STZ) and alloxan to pregnant animals leads to the selective destruction of pancreatic cells, thus generating a maternal diabetic state related to type 1 diabetes during pregnancy.

Both rat and human placentas are hemochorial (Yan et al., 2005), meaning maternal blood is no longer contained in blood vessels but is in direct contact with fetal trophoblasts that also embed fetal capillaries in the labyrinthine trophoblast zone (Zhang et al, 1998). This similarity, combined with the short duration of their pregnancies and their easy maintenance, are advantages of using rats in diabetes studies. Despite the existence of developmental and morphological differences in the placentas of rodents and women, there are many similarities in the alterations induced by maternal diabetes in the placenta from diabetic patients and diabetic experimental models (Kuhn et al., 1990; Desoye

and Shafrir 1994; Georgiades et al., 2002; Radaelli et al., 2003; Lappas et al., 2004; Jawerbaum and Gonzalez, 2006).

Placentomegalia is observed in various mild and severe chemically-induced diabetic experimental models (Diamant et al., 1982; Eriksson et al., 1989; Capobianco et al., 2005; Suwaki et al., 2007; Acar et al., 2008). Structural, functional, and developmental abnormalities are found in the placentas of streptozotocin induced diabetic rodents (Barash et al., 1985; Padmanabhan and al-Zuhair 1990; Caluwaerts et al., 2000). Moreover, array studies have shown an aberrant gene expression pattern in placentas from streptozotocin-induced diabetic mice (Yu et al., 2008). Increased amounts of lipids, glycogen, and DNA characterize the placentas from streptozotocin-induced diabetic rodents (Diamant et al., 1982; Shafrir and Barash, 1987). Glucose transfer through the placenta increases linearly with maternal glucose in streptozotocin-induced diabetic rats (Herrera et al., 1985). Some of these changes mirror the changes seen in human diabetic placentas, indicating that this is a reasonable model for the exploration of human diabetic pregnancy.

Proper development of an organism requires an integration of cell cycle exit and differentiation pathways. Proliferation is positively regulated by cyclin-dependent kinases (CDKs) (Zhang et al., 1998). Cell cycle arrest and activation of differentiation processes require inhibition of CDKs by CDK inhibitors (CKIs) (Rao et al., 1994; Skapek et al., 1995, 1996). p57 is a CDK inhibitor that has the ability to bind with a variety of cyclin-CDK complexes and to inhibit their kinase activities. Based on their sequence homology and specificity of action, CKIs are divided into two distinct families: INK4 and Cip/Kip (Sherr et al., 1999). INK4 family members include p15, p16, p18, and p19, and specifically inhibit the activity of CDK4 and CDK6. Cip/Kip members include p21, p27, and p57 (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Polyak et al., 1994; Toyoshima and Hunter, 1994; Lee et al., 1995). This family causes cells to arrest in the G1 phase. Even though p21 and p27 are widely expressed, p57 is expressed in a tissue-specific manner (Lee et al., 1995; Matsuoka et al., 1995). p57 inhibits cyclin A- and E-associated CDKs and therefore regulates the G1/S transition and the completion of S phase (Lee et al., 1995), and is primarily expressed in terminally differentiated cells (Yan et al., 1997). Recently, p57 was identified among the paternally imprinted (maternally expressed) genes in humans and mice (Matuoka et al., 1996). p57 has a restricted tissue distribution with high levels of expression in human placentas, suggesting that it has a specialized function in cell cycle control during implantation and placental development (Lee et al., 1995; Chilosi et al., 1997, 1998).

p57 deficient mice displayed organomegaly and abdominal wall defects (Zhang et al., 1997), increased apoptosis and delayed differentiation during mouse development (Yan et al., 1997), immaturity of some tissues, and severe growth retardation (Takahashi et al., 2000a). In a study done by Takahashi et al. (2000b), placentas of $p57^{-/-}$ mouse embryos showed hyperplasia involving both labyrinthine trophoblasts and spongiotrophoblasts. p57 may function in the proper development of labyrinthine and spongio-trophoblasts by pathways that are not involved with the regulation of CDK activities.

Developmental defects in the placenta were observed in another study regarding p27 and p57 knockout mice. Since the labyrinthine trophoblast zone was less vascularized and contained more trophoblasts compared with that in wild-type mice, it was suggested that the absence of these CDK inhibitors might alter the differentiation of trophoblasts in the labyrinthine zone, allowing them to proliferate inappropriately (Zhang et al., 1998). p57 expression is markedly reduced in patients with malignant trophoblastic neoplasms, which can result in spontaneous abortions and preterm deliveries (Chilosi et al., 1998), and deficiency in p57 expression induced preeclampsia-like symptoms in mice (Kanayama et al., 2002).

Recent studies suggest that Cip/Kip proteins play additional roles outside of the nucleus (Coqueret, 2003). p57 modulates subcellular localization of LIMK, a serine/threonine kinase involved in the regulation of actin filaments (Yokoo et al., 2003). Although placental development depends on careful coordination of trophoblast proliferation and differentiation, little is known about the mitotic regulators that play key roles in synchronizing these events (Genbacev et al., 2000).

Since we observed different expression patterns of the cell cycle protein proliferating cell nuclear antigen (PCNA) in diabetic rat placentas compared to control rat placentas, we hypothesized that the mitotic activity of the rat placenta under diabetic conditions might show alterations during pregnancy. This study was to investigate the expression of CDK inhibitor p57 during the growth and differentiation of normal and diabetic rat placentas.

Materials and methods

Animals

Sixty-four female and 32 male adult Wistar rats (*Rattus norvegicus*) weighing 200–250 g were used in the study and none of them had mated previously. All rats were allowed free access to a standard rat laboratory diet and tapwater and were maintained on a 12:12 h light/dark cycle. Two females and one male were kept overnight in one cage. A sperm-positive vaginal smear observed on the following morning was considered to indicate successful copulation. The sperm-positive day was designated as day 0 of pregnancy. Pregnant animals were caged separately and were randomly assigned to either the control or diabetic groups. Diabetes was induced in the appropriate group of rats by a single injection of STZ on the first day of pregnancy. Rats in

the diabetic-induced group were injected intraperitoneally with a single dose of 50 mg/kg of STZ (Sigma-Aldrich Co., St. Louis, MO, USA; 63178) freshly prepared in 0.1M citrate buffer (pH 4.5). Animals in the control group received an identical volume of citrate buffer.

Blood glucose concentrations were measured 48 h after STZ injection to confirm that diabetes had been induced. Blood samples were obtained from the tail vein. Animals exhibiting blood glucose levels higher than 200 mg/dl (Accu-Chek Active test strips, Roche Diagnostic GmbH Mannheim, Germany, 3146332) were considered to be diabetic. Animals that did not develop diabetes were excluded from the experiments.

Control and STZ diabetic mothers were anesthetized with ether, sacrificed by cervical dislocation, and embryos, fetuses, and placentas were taken on days 11, 13, 17, and 21 of gestation. Fetal and placental weights were recorded (Acar et al., 2008). The experimental protocols were approved by the Animal Care and Usage Committee of Akdeniz University and were in accordance with the guidelines of the International Association for the Study of Pain.

Tissue processing

Placental samples were fixed in Holland's fixative consisting of 4% formaldehyde, 5ml glacial acetic acid, 4 g picric acid, and 2.5 g cupric acetate in 100 ml distilled water. After dehydration in ethanol, tissues were cleared in xylene and embedded in paraffin wax. Subsequently, serial 5-mm-thick sections were cut and mounted on poly-L-lysine coated slides (Sigma-Aldrich, St. Louis MO, USA, P8920).

p57 immunohistochemistry

The immunohistochemical procedure has been described elsewhere (Acar et al., 2008). Briefly, sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. To unmask antigens, an antigen retrieval procedure was performed by treating the samples in 10 mM citrate buffer, pH 6.0, in a microwave oven at 750W for 5 min, twice. After cooling for 20 min at room temperature, the sections were washed in phosphate buffered saline (PBS; pH 7.4). Endogenous peroxidase activity was blocked by incubation in methanol containing 3% H2O2 for 30 min and sections were then washed with PBS three times. Afterwards, sections were incubated in a blocking serum (Ultra UV Block, LabVision Corporation, Fremont, CA, USA; TA-125UB) for 7 min at room temperature to block non-specific binding. Rabbit polyclonal anti-p57 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-8298) was applied at a 1:200 dilution for 1 h at room temperature. Negative controls were performed by replacing the primary antibody with rabbit serum. Sections were rinsed three times in PBS and incubated with biotinylated secondary antibody (DAKO, Glostrup, Denmark; LSAB-2 system HRP K0609-ready to use) and a peroxidase labeled streptavidin (DAKO, Glostrup, Denmark; LSAB-2 system HRP K0609-ready to use), respectively, for 30 min, with each of the steps being followed by three rinses in PBS. Peroxidase activity was visualized with 3-amino-9-ethylcarbazol (AEC) (Lab Vision Corporation, Fremont, CA, USA; TA-125-HAC) chromogen in large-volume AEC substrate (Lab Vision Corporation, Fremont, CA, USA; TA-125 HAS) as one drop chromogen in 1ml substrate for 4 min. All dilutions were performed using PBS unless otherwise stated. Sections were counterstained with hematoxylin, dehydrated, mounted in Kaiser's glycerin gelatin (Merck, NJ, USA; OB514196), and examined by light microscopy. Tissue sections from different pregnancy days were evaluated for protein localization and immunolabeling intensity. All samples were treated with exactly the same protocol.

H-SCORE and semi-quantitative evaluations

The evaluation of the immunohistochemical labeling of p57 in samples from both diabetic and control groups was performed using H-SCORE (Celik-Ozenci et al., 2004). Briefly, sections were evaluated using an Axioplan microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. Three randomly selected slides, each of five different fields (two maternal and three fetal zones) at 200X magnification, were evaluated for immunohistochemical labeling of p57. The evaluations were recorded as percentages of labeled cells of all types in each of four intensity categories, denoted as 0 (no labeling), 1+ (weak labeling but detectable above control), 2+ (distinct labeling), and 3+ (intense labeling). For each tissue, an H-SCORE value was derived by summing the percentages of cells that were labeled at each intensity multiplied by the weighted intensity of the labeling: H-SCORE=Σ Pi (i+1), where i is the intensity score and Pi is the corresponding percentage of the cells. Two observers blind to the experimental groups performed the H-SCORE evaluations, and the average score was used. The distribution of immunoreactive cells in all experimental groups was also determined semi-quantitatively: 0=negative; (+)=weak positive; +=positive; ++=strong positive; +++=very strong positive.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

Protein extraction and immunoblot analysis were performed as described previously (Hahn et al., 2001). Four placenta samples for each group were weighed and put into a homogenization buffer supplemented with CompleteR protease inhibitor cocktail (Boehringer, Mannheim, Germany). After homogenization, samples were centrifuged at 10,000g for 10 min. Supernatants

were collected and stored at -70°C.

The protein concentration was determined by Lowry assay (Lowry et al., 1951), and 50 μg of protein was applied per lane. Samples were subjected to SDS polyacrylamide gel electrophoresis (30% acrylamide in 12% gel) at 100 V for approximately 1.5 h and were then transferred onto nitrocellulose membranes (Pharmacia, Kirkland, Quebec, Canada) in a buffer containing 0.2 mol/L glycine, 25 mmol/L Tris, and 20% methanol overnight, at 4°C, under 32 V and 65 A. The membranes were blocked for 1 h with 5% non-fat dry milk (BioRad Laboratories, Hercules, CA, USA) and 0.1% Tween 20 (Sigma-Aldrich, St Louis, MO, USA) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2-7.4) at 4°C. Membranes were incubated with a 1:200 dilution of rabbit polyclonal anti-p57 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-8298), overnight. After the washing steps, the membranes were further incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad Laboratories, Hercules, CA, USA; 170-6515), diluted to 1:2500, for 1 h at room temperature.

Immunolabeling was visualized by using the chemiluminescence-based SuperSignal CL HRP Substrate System (Pierce, Rockford, IL, USA), prepared according to the manufacturer's instructions, and the membranes were exposed to Hyperfilm (Amersham, Bucks, UK; 28906837). Membranes were also labeled by an identical protocol for binding of a 1:5000 dilution of an anti-mouse ß-actin antibody (Abcam, Cambridge, UK; ab 6276-100) as an internal control to confirm equal loading of the samples. After the washing steps, the membranes were further incubated with goat anti-mouse IgG horseradish peroxidase conjugate (BioRad Laboratories, Hercules, CA, USA; 170-6516), diluted to 1:5000, for 1 h at room temperature. The bands were quantified using NIH image analysis software (Image J Version 1.36b, National Institutes of Health, USA).

Statistical analysis

The following comparisons between control and diabetic groups were performed by a Mann–Whitney rank sum test and Wilcoxon signed-rank test: H-SCORE values for p57 immunohistochemistry and p57 Western blot band values. Mann–Whitney rank sum test comparisons were made between control and diabetic group samples taken from the same day. Wilcoxon signed-rank test comparisons were made between days for the same group, i.e. between control group day 11 and control group day 13. Values are presented as mean ± SEM. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences 10.0 for Windows, Chicago, IL, USA).

Results

Immunohistochemical staining of p57

The distribution pattern of p57 immunolabeling intensity in control and diabetic group placentas is summarized in Table 1.

p57 immunostainings were in nuclei in both control and diabetic placentas. On day 11 of pregnancy, p57 immunolabeling was seen in a few cell types in the placenta of the control group. Most of the positive cells were labyrinthine trophoblasts, labyrinthine fetal vessel endothelial cells, and spongiotrophoblasts. A few giant cells showed strong positive immunopositivity. In Figure 1a positive labyrinthine trophoblasts and strong positive labyrinthine fetal vessel endothelial cells are seen. On the same day in the diabetic group the positive stainings for cell types and region were similar to the control group; however, the staining intensity of the p57 positive cells was less in the labyrinthine trophoblast zone when compared to control group. Weak positive labyrinthine trophoblasts and labyrinthine fetal vessel endothelial

Table 1. Semi-quantitative scoring of p57 immunostaining intensities in control and diabetic group rat placenta zones.

	Allantois				Labyrinth		Junctional zone		
Gestation days	VEC	PEC	AVEC	AMT	LFVEC	LT	ST	Gly. C	GC
Control									
11	0	0	0	0	++	+	+	x	++
13	0	0	0	0	++	++	++	x	+++
17	++	(+)	+	0	+	++	+	+	++
21	(+)	0	+++	0	+++	+++	+++	x	+++
Diabetic									
11	0	0	0	0	(+)	(+)	+	x	++
13	+	0	(+)	0	+++	+++	+++	x	+++
17	+	+	+	0	(+)	+	(+)	(+)	++
21	0	0	++	0	++	++	++	0	+++

0: negative; (+): weak positive; +: positive; ++: strong positive; +++: very strong positive; x: this zone doesn't exist; AMT: allantoic mesenchymal tissue; AVEC: allantoic vessel endothelial cell; CDK: cyclin-dependent kinase; CKI: cyclin-dependent kinase (CDK) inhibitor; GC: giant cell; Gly C: glycogen cell; LFVEC: labyrinthine fetal vessel endothelial cell; LT: labyrinthine trophoblast; PCNA: proliferating cell nuclear antigen; PEC: parietal endoderm cell; ST: spongiotrophoblast; VEC: visceral endoderm cell.

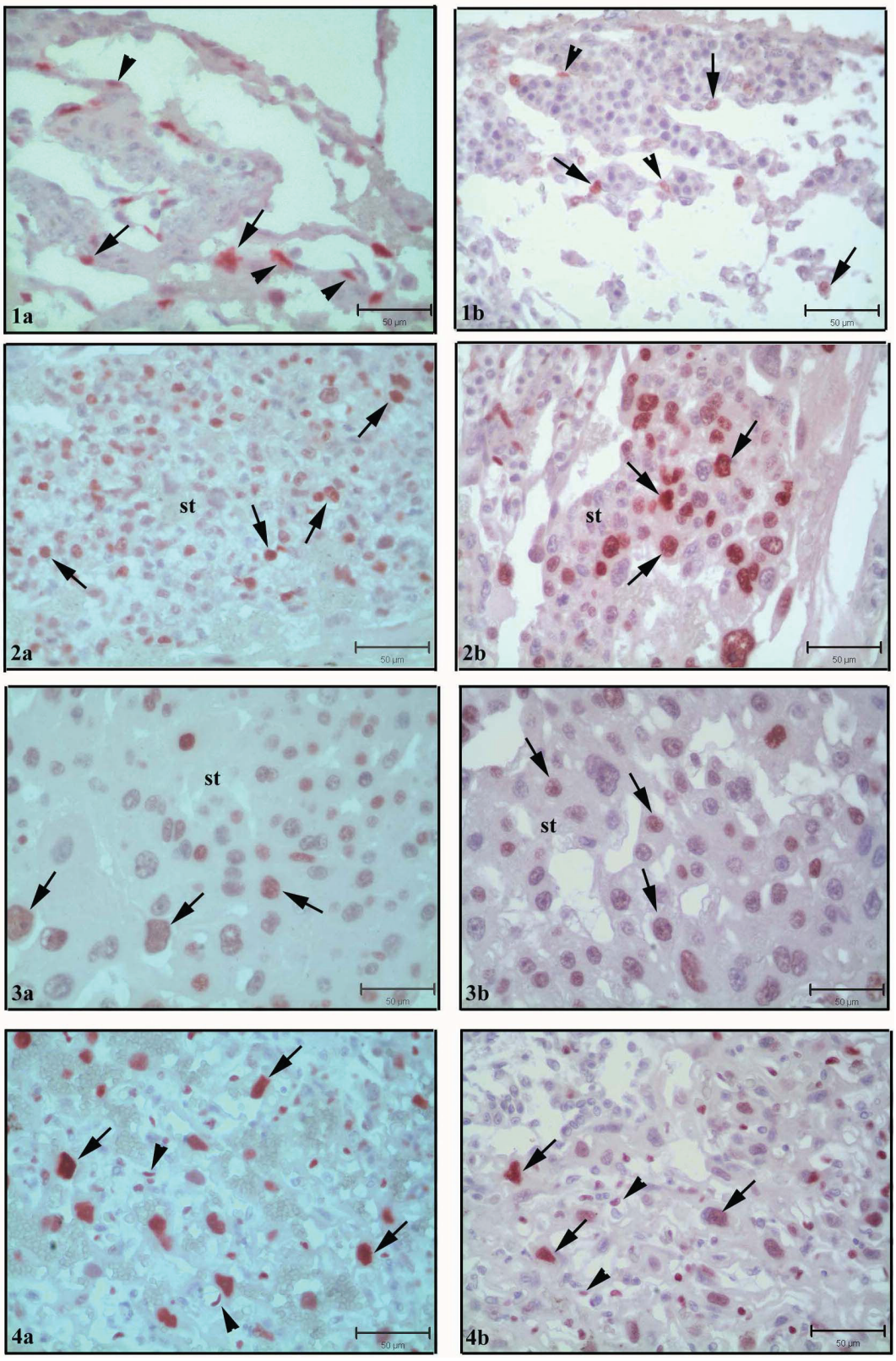


Fig. 1. p57 immunolabeling on day 11 of pregnancy. **a.** Positive p57 immunolabeling in labyrinthine trophoblasts (arrows) and strong positive p57 immunolabeling in labyrinthine fetal vessel endothelial cells (arrow heads) on day 11 control group rat placenta. **b.** p57 immunolabeling intensity was weak positive in labyrinthine trophoblasts (arrows) and labyrinthine fetal vessel endothelial cells (arrow heads) in the diabetic group, which is less than the same day control group. Scale bar: 50 μm.

Fig. 2. p57 immunolabeling on day 13 of pregnancy. **a.** Strong positive p57 immunolabeling in spongiotrophoblasts (arrows) on day 13 control group rat placenta. **b.** p57 immunolabeling intensity was greater (very strong positive) in diabetic group spongiotrophoblasts (arrows) compared to the control group on the same day. st: spongiotrophoblast. Scale bar: 50 μm.

Fig. 3. p57 immunolabeling on day 17 of pregnancy. **a.** p57 immunolabeling intensity was positive in spongiotrophoblasts (arrows) in control group placentas on day 17 of pregnancy. **b.** p57 immunolabeling intensity was less (weak positive) in diabetic group spongiotrophoblasts (arrows) compared to the same day control group. st: spongiotrophoblast. Scale bar: 50 μm.

Fig. 4. p57 immunolabeling on day 21 of pregnancy. **a.** p57 immunolabeling intensity in the control group on day 21 of pregnancy. Very strong positive p57 immunolabeling intensity in labyrinthine trophoblasts (arrows) and labyrinthine fetal vessel endothelial cells (arrow heads). **b.** Weaker (strong positive) p57 immunolabeling intensities were observed in both the labyrinthine trophoblasts (arrows) and labyrinthine fetal vessel endothelial cells (arrow heads) in the diabetic group on day 21 of pregnancy compared to the same day control group. Scale bar: 50 μm.

cells are seen in Figure 1b.

On day 13 of pregnancy, in both the control and diabetic groups, p57 immunostaining intensity was stronger compared to control and diabetic groups on day 11 of pregnancy. Positively stained cells were mostly labyrinthine trophoblasts, spongiotrophoblasts, and giant cells. Immunoreactivity was strong positive in spongiotrophoblasts (Fig. 2a). In diabetic group placentas p57 immunostaining was more intense compared to the control group. Spongiotrophoblasts were very strong positive (Fig. 2b).

On day 17 of pregnancy p57 immunostaining intensity decreased both in control and diabetic group placentas compared to day 13 of pregnancy. Immunostaining intensity was positive in allantoic vessel endothelial cells, labyrinthine fetal vessel endothelial cells, glycogen cells, and spongiotrophoblasts. Positive p57 immunostaining is seen in spongiotrophoblasts in Figure 3a. On the same day diabetic group placentas exhibited weaker immunoreactivity in almost every stained cell compared to the control group. Immunoreactivity in spongiotrophoblasts in the diabetic group was less than the control group's weak positive reactivity (Fig. 3b).

On day 21 of pregnancy the control group's p57 positive cell number and staining intensity was greater in all zones of the placenta than on days 11, 13, and 17 in the control and diabetic groups' placentas. Very strong positive p57 immunostaining is seen in labyrinthine trophoblasts and labyrinthine fetal vessel endothelial cells in control group placentas (Fig. 4a). On the other hand, on day 21 the diabetic group's p57 positive cell number and immunostaining intensity was less than the day 21 control group. Strong positive immunostaining is seen in labyrinthine trophoblasts and labyrinthine fetal vessel endothelial cells in diabetic group placentas (Fig. 4b).

H-SCORE analysis revealed that immunolabeling intensity in the control group placentas was higher than that in the diabetic group placentas on days 11, 17, and 21 of pregnancy. On day 13 of pregnancy p57 immunolabeling intensity was higher in the diabetic group compared to the control group placentas. All stained cell numbers and immunolabeling intensity in the control group on day 21 of pregnancy was greater when compared to the control and diabetic groups on pregnancy days 11, 13, and 17. H-SCORE values for control and diabetic groups are shown in Table 2. The differences between control and diabetic groups were statistically significant on days 11 and 21.

A Wilcoxon signed rank test showed that in the control group the changes in p57 immunolabeling intensity was statistically significant ($p<0.05$) between: day 11 and days 13 and 21; day 13 and day 17; and, day 17 and day 21. In the diabetic group, the changes in p57 immunolabeling intensity was statistically significant ($p<0.05$) between: day 11 and day 13; and, between day 13 and days 17 and 21 (not shown).

Western blot results

Consistent with p57 immunohistochemistry H-SCORE results, p57 protein amounts on days 11, 17, and 21 of pregnancy were higher in control group placentas; these differences were statistically significant on days 11 and 21 ($p=0.029$, $p<0.001$ respectively) but not on day 17 ($p=1$). On day 13 of pregnancy the p57 protein amount appeared to be higher in diabetic group placentas compared to control group placentas; this difference was statistically significant ($p=0.017$) (Fig. 5a, b).

Table 2. H-SCORE values of p57 immunostaining in control and diabetic group rat placentas on days 11, 13, 17, and 21 of pregnancy.

Gestation Days	CONTROL	DIABETIC
H-SCORE VALUES		
11	104,455±2,201	66,545±3,627
13	113,909±3,204	121,545±8,218
17	90,818±6,923	71,364±7,148
21	131,273±9,135	73,545±6,338

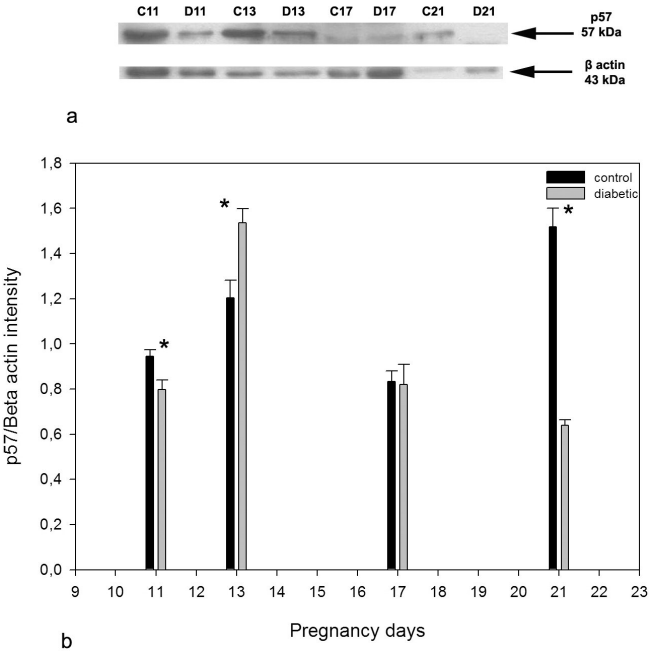


Fig. 5. a. Western blot results of p57 in control and diabetes-induced groups. ß-actin was used as an internal standard. **b.** Quantitative analysis of p57 Western blot data. The values in the bar graphs were obtained by dividing the protein intensity value by its corresponding ß-actin intensity value. The differences seen between groups on days 11, 13, and 21 were statistically significant. Abbreviations: C11, C13, C17, and C21 for the control group on days 11, 13, 17, and 21 of pregnancy, respectively; D11, D13, D17, and D21 for the diabetic group on parallel days of pregnancy, respectively.

Discussion

Proper development of the placenta is dependent on the formation of trophoblasts. Trophoblast proliferation is tightly controlled, because it is important for normal proliferation, differentiation, and function of the decidual trophoblastic unit, which are necessary for successful implantation. Therefore, there must be a balance between the positive cell cycle factors and the negative cell cycle inhibitors (Zhang et al., 1997).

The altered metabolic environment in maternal diabetes during pregnancy interferes with normal fetal development (Padmanabhan and al-Zuhair, 1987). Abnormally elevated glucose levels in maternal blood, which lead to increased glucose transport to the embryo, are responsible for the teratogenic effects of maternal diabetes. Consequently, the expression of genes that control essential developmental processes is disturbed (Loeken, 2005). Deregulation of the cell cycle can cause uncontrolled cell division, resulting in the development or progression of trophoblastic disease. Since p57 is a cell cycle inhibitor and tumor suppressor, lack of p57 activity can lead to a loss of cell cycle control and hyperproliferation (Zhang et al., 1997).

STZ-induced diabetic rats are characterized by placentomegaly and varying degrees of fetal growth retardation (Robinson et al., 1988; Acar et al., 2008). Placental enlargement is also a feature of human diabetic pregnancies. Whereas macrosomia often occurs in infants of diabetic women, growth retardation is almost a rule in spontaneous and experimental diabetes in animals. The functional significance of placentomegaly in the presence of maternal diabetes in rats and other species is unclear (Husain et al., 2001), and it is not clear when the growth inhibition starts or how placental pathology might affect fetal growth in maternal diabetes (Padmanabhan and Shafiullah, 2001). Some researchers hypothesize that hyperglycemia leads to a relative immaturity of rat placentas by providing a stimulus for continuous growth and cell division-delayed maturation (Gewolb et al., 1986).

Takahashi et al. (2000b) reported that both $p57^{-/-}$ and $p57^{+/-}$ mice had heavier placentas compared to wild type mice but detected no difference in the body weights of embryos between the genotypes. Histopathologic analysis of placentas from homozygote knock-out mice showed that labyrinthine- and spongio-trophoblasts had prominent proliferation, resulting in thickened placentas and narrowed interlabyrinthine trophoblast spaces. Fibrin deposition of the intervillous space was also observed. There were no statistically significant increases in glycogen or giant cells in the knock-out group.

To elucidate the mechanism underlying trophoblastic hyperplasia seen in the placentas of mice lacking p57 expression, Matsuura et al. (2002) studied the expression of vascular endothelial growth factor (VEGF), and they showed higher VEGF mRNA and protein levels in knock-out placentas compared to wild-type placentas. They concluded that VEGF is involved in the hyperplasia that occurs in placentas of p57 null embryos.

In another study, Zhang et al. (1998) conducted histopathological observations on $p27^{-/-}p57^{+/-}$ mice. In placentas derived from $p57^{+/-m}$ single or $p27^{-/-}$ $p57^{+/-m}$ double mutants, the labyrinthine trophoblast zone was less vascularized and contained more trophoblasts than those from wildtype or $p27^{-/-}$ mice. The diameter of most mutant fetal capillaries was reduced to the size of a single fetal red blood cell, leading to the appearance of less vascularization. They suspected that the absence of these CKIs might alter the differentiation of trophoblasts in the labyrinthine trophoblast zone, allowing them to proliferate inappropriately.

Knox and Baker (2007) showed the localization of p57 in wild-type mouse placenta on embryonic days 10 (e10) and 17 (e17) with the in situ hybridization method. At e10, there was staining of the labyrinthine- and spongio-trophoblast layers, as well as the overlying trophoblast giant cells. At e17, there was intense staining in the labyrinthine trophoblast, as well as in clusters of cells within the spongiotrophoblast layer (Knox and Baker, 2007). We also observed p57 expression in the labyrinthine trophoblast, spongiotrophoblast, and giant cell layers on day 11 of pregnancy. Similarly, on day 17 of pregnancy there was p57 immunoreactivity in both the labyrinthine- and spongio-trophoblasts.

Kim et al. (2005) studied the expressions of p27 and p57 in mouse placenta on days 12, 14, and 16 of pregnancy. They reported the immunolocalization of p57 in both the nucleus and cytoplasm of decidua, spongio- and labyrinthine-trophoblast cells. According to their Western blot results, p57 expression increased from day 12 to day 14 and then decreased on days 16 and 18. They concluded that p27 may play a role in the late period of mouse placental development and p57 may play a role in the middle period of mouse placental development. Consistent with this study, we observed an increase in p57 protein amounts from day 11 to day 13 of pregnancy. Then there was a decrease on day 17; afterwards, p57 expression increased in the control group on day 21 of pregnancy.

Pedersen and Molsted-Pedersen (1979) noted that in diabetic pregnancies fetal growth is retarded early in pregnancy, leading to low birth weight and possibly a higher incidence of congenital malformations. In our previous study (Acar et al., 2008), embryo weights in diabetic groups on all studied days (days 11, 13, 17, and 21 of pregnancy) were lower than that of the control group embryos on all days examined (not represented here). We also showed that placental weights in the diabetic groups were less than in control groups on days 11 and 13 of pregnancy, while higher placental weights were recorded on days 17 and 21 of pregnancy; these results were consistent with previous studies (Padmanabhan and al-Zuhair, 1987; Robinson et al., 1988; Caluwaerts et al., 2000; Padmanabhan and Shafiullah, 2001). There was an increase in placental weights after day 17 of rat pregnancy, suggesting that

this increase might be due to increased protein synthesis and cell proliferation (Acar et al., 2008). These studies indicate that diabetes may contribute to fetal growth retardation and placentomegaly starting in the last third of pregnancy in rats. Robinson et al. (1988), reported that on days 14 and 16 of pregnancy placental weights of animals in the diabetic group were less than those in the control group, but the weight of placentas increased after day 18 of pregnancy. In this study, they also showed that in the early stages of pregnancy placentas of animals in the diabetic group had levels of protein, RNA, DNA, and cell numbers that were similar to levels seen in the control group. But after day 18 of pregnancy, diabetic placentas had more protein, RNA, DNA, and cells compared to control group placentas. Glycogen storage increased in the diabetic group placentas starting from day 16 of pregnancy, but decreased slowly with advancing pregnancy in control group placentas.

We showed abnormal expression patterns of PCNA in diabetic rat placenta in our previous study (Acar et al., 2008). On days 11 and 13 of pregnancy, PCNA protein amounts were higher in control group placentas; however, these differences were not statistically significant. We also observed greater PCNA immunolabeling intensity in diabetic group placentas on days 17 and 21 of pregnancy when compared to the controls, with no statistically significant difference. We had suggested that higher PCNA protein intensity/amounts in diabetic groups on days 17 and 21 of pregnancy may arise from spongiotrophoblasts, because this cell type was observed to maintain its very strong immunolabeling intensity until day 21 of pregnancy. This is contradictory to the report of Mark et al. (2006). They emphasized that during the final third of rat pregnancy the basal zone exhibits apoptosis and shows little, if any, growth, whereas the labyrinthine trophoblast zone grows substantially and apoptosis is very low over this period. It is possible that spongiotrophoblasts are affected much more than the labyrinthine trophoblasts under diabetic conditions.

Trophoblast cells at the periphery of the placenta undergo terminal differentiation to form polyploid giant cells in rodents. The formation of the terminally differentiated outer layer of trophoblast cells is critical for implantation and for the establishment of connections between the placenta and the maternal vasculature. According to our study, p57 expression was strong/very strong positive in all studied pregnancy days in control group placentas. However expression was lower in diabetic group placentas compared to control group placentas. The less differentiated giant cells in diabetic group placentas may not be able to invade the endometrium deeply enough at the beginning of implantation.

Cell proliferation is internally regulated by a balance of cell cycle activator proteins and cell cycle inhibitor proteins. As most cells differentiate during development, they begin to express CDK inhibitors to arrest cell cycle progression. Genbacev et al. (2000) showed that the percentage of cytotrophoblasts that expressed cell cycle inhibitors correlated positively with increased gestational age in human placentas; they speculated that this population was composed of differentiating cytotrophoblasts.

Goffin et al. (2003) examined the localization of p57 in first trimester human placentas. According to their immunohistochemistry results, there was no reaction in syncytium, villous cytotrophoblasts, and proliferative extravillous cytotrophoblasts, but they found reaction in invasive extravillous cytotrophoblasts. Korgun et al. (2006) investigated the immunolocalization of some cell cycle markers in first trimester human placentas, and p57 was shown to localize predominantly in villous and extravillous cytotrophoblasts. They commented that p57 expression in extravillous cytotrophoblasts, which invade the decidua deeply, inhibit cell cycle progression and allow the cells to differentiate and change their phenotype, accounting for the loss of mitogenic potential of these cells.

p57 may be important as a tumour supressor during pregnancy (Takahashi et al., 2000b). It has been shown that p57 expression is markedly reduced in patients with malignant trophoblastic neoplasms which can result in spontaneous abortions and preterm deliveries (Chilosi et al., 1998). Fukunaga et al. (2002, 2004) reported p57 to be at high frequency in normal placentas, but absent or at low frequency in diploid and tetraploid complete hydatidiforms. Currently, there have not been any studies examining p57 expression in diabetic human placentas. However, because we observed abnormal expression of p57 in our streptozotocin-induced diabetic model, and because many alterations in these types of experimental models are similar to those found in human diabetic placentas (Jawerbaum and White, 2010), our work could point to an area that deserves to be explored in human tissue samples.

In brief, we observed an abnormal expression pattern of PCNA and p57 in our previous and current study, respectively, concerning diabetic rat placentas. Since p57 controls the cell cycle exit and is expressed less in diabetic placentas on days 17 and 21 of pregnancy, diabetic placentas may continue to proliferate on these days. Less p57 expression in diabetic placentas on days 17 and 21 of pregnancy compared to control placentas may explain the reason why diabetic placentas are heavier and bigger.

In conclusion, small placentas in the early stages of pregnancy and placentomegaly in later stages of pregnancy in diabetic rats may occur as a result of disturbed cell cycle pathways with the participation of p57 and other mitotic regulators, like PCNA, as we previously showed. There are relatively few studies on the role of mitotic regulators involved in rat placental development. Our research may provide the basis for further investigations to improve our understanding of factors leading to abnormal placentation; however, more detailed studies are required to explain the molecular mechanisms of diabetic placentation.

Acknowledgements. This study is a part of the Master's thesis of Nuray Acar and was supported by a grant from the Akdeniz University Research Fund (2004.02.0122.001), Antalya, Turkey.

References

Acar N., Korgun E.T., Cayli S., Sahin Z., Demir R. and Ustunel I. (2008). Is there a relationship between PCNA expression and diabetic placental development during pregnancy? Acta Histochem. 110, 408-417.

Barash V., Gutman A. and Shafrir E. (1985). Fetal diabetes in rats and its effect on placental glycogen. Diabetologia 28, 244-249.

Caluwaerts S., Pijnenborg R., Luyten C. and Van Assche F.A. (2000). Growth characteristics of diabetic rat ectoplacental cones *in vivo* and *in vitro*. Diabetologia 43, 939-945.

Capobianco E., Jawerbaum A., Romanini M.C., White V., Pustovrh C., Higa R., Martinez N., Mugnaini M.T., Sonez C. and Gonzalez E. (2005). 15-Deoxy-delta(12,14)-prostaglandin J2 and peroxisome proliferator-activated receptor γ (PPAR γ) levels in term placental tissues from control and diabetic rats: modulatory effects of a PPAR γ agonist on nitridergic and lipid placental metabolism. Reprod. Fertil. Dev. 17, 423-433.

Celik-Ozenci C., Akkoyunlu G., Korgun E.T., Savas B. and Demir R. (2004). Expressions of VEGF and its receptors in rat corpus luteum during interferon alpha administration in early and pseudopregnancy. Mol. Reprod. Dev. 67, 414-423.

Chilosi M., Doglioni C., Menestrina F., Lestani M., Piazzola E., Benedetti A., Pedron S., Montagna L., Pizzolo G., Mariuzzi G.M. and Janossy G. (1997). Expression of extracellular matrix molecules, proliferation markers and cyclin-dependent kinase inhibitors in inflamed tissues. Biochem. Soc. Trans. 25, 524-528.

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). Differential expression of p57kip2, a maternally imprinted Cdk inhibitor, in normal human placenta and gestational trophoblastic disease. Lab. Invest. 78, 269-276.

Coqueret O. (2003). New roles for p21 and p27 cell-cycle inhibitors: A function for each cell compartment? Trends Cell Biol. 13, 65-70.

Desoye G. and Shafrir E. (1994). Placental metabolism and its regulation in health and diabetes. Mol. Aspects. Med. 15, 505-682.

Diamant Y.Z., Metzger B.E., Freinkel N. and Shafrir E. (1982). Placental lipid and glycogen content in human and experimental diabetes mellitus. Am. J. Obstet. Gynecol. 144, 5-11.

el-Deiry W.S., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer W.E., Kinzler K.W. and Vogelstein B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell 75, 817-825.

Eriksson U.J., Bone A.J., Turnbull D.M. and Baird J.D. (1989). Timed interruption of insulin therapy in diabetic BB/E rat pregnancy: effect on maternal metabolism and fetal outcome. Acta Endocrinol. (Copenh) 120, 800-810.

Fukunaga M. (2002). Immunohistochemical characterization of p57KIP2 expression in early hydatidiform moles. Hum. Pathol. 33, 1188-1192.

Fukunaga M. (2004). Immunohistochemical characterization of p57Kip2 expression in tetraploid hydropic placentas. Arch. Pathol. Lab. Med. 128, 897-900.

Genbacev O., McMaster M.T. and Fisher S.J. (2000). A repertoire of cell cycle regulators whose expression is coordinated with human cytotrophoblast differentiation. Am. J. Pathol. 157, 1337-1351.

Georgiades P., Ferguson-Smith A.C. and Burton G.J. (2002). Comparative developmental anatomy of the murine and human definitive placentae. Placenta. 23, 3-19.

Gewolb I.H., Merdian W., Warshaw J.B. and Enders A.C. (1986). Fine structural abnormalities of the placenta in diabetic rats. Diabetes 35, 1254-1261.

Goffin F., Munaut C., Malassine A., Evain-Brion D., Frankenne F., Fridman V., Dubois M., Uzan S., Merviel P. and Foidart J.M. (2003). Evidence of a limited contribution of feto-maternal interactions to trophoblast differentiation along the invasive pathway. Tissue Antigens 62, 104-116.

Gu Y., Turck C.W. and Morgan D.O. (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. Nature 366, 707-710.

Hahn D., Blaschitz A., Korgun E.T., Lang I., Desoye G., Skofitsch G. and Dohr G. (2001). From maternal glucose to fetal glycogen: expression of key regulators in the human placenta. Mol. Hum. Reprod. 7, 1173-1178.

Harper J.W., Adami G.R., Wei N., Keyomarsi K. and Elledge S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75, 805-816.

Herrera E., Palacin M., Martin A. and Lasuncion M.A. (1985). Relationship between maternal and fetal fuels and placental glucose transfer in rats with maternal diabetes of varying severity. Diabetes 34 (Suppl 2), 42-46.

Husain S.M., Frost R. and Mughal Z.M. (2001). Effect of Diabetes mellitus on rat placenta cellularity. Early Hum. Dev. 60, 207-214.

Jawerbaum A. and Gonzalez E. (2006). Diabetic pregnancies: the challenge of developing in a pro-inflammatory environment. Curr. Med. Chem. 13, 2127-2138.

Jawerbaum A. and White V. (2010). Animal models in diabetes and pregnancy. Endocr. Rev. 31, 680-701.

Kanayama N., Takahashi K., Matsuura T., Sugimura M., Kobayashi T., Moniwa N., Tomita M. and Nakayama K. (2002). Deficiency in p57kip2 expression induces preeclampsia- like symptoms in mice. Mol. Hum. Reprod. 8, 1129-1135.

Kim S.T., Lee S.K. and Gye M.C. (2005). The Expression of Cdk Inhibitors p27kip1 and p57kip2 in Mouse Placenta and Human Choriocarcinoma JEG-3 Cells. Placenta. 26, 73-80.

Knox K.S and Baker J.C. (2007). Genome-wide expression profiling of placentas in the p57Kip2 model of pre-eclampsia. Mol. Human Reprod. 13, 251-263.

Korgun E.T., Celik-Ozenci C., Acar N., Cayli S., Desoye G. and Demir R. (2006). Location of cell cycle regulators cyclin B1, cyclin A, PCNA, Ki67 and cell cycle inhibitors p21, p27 and p57 in human first trimester placenta and deciduas. Histochem Cell Biol. 125, 615-624

Kuhn D.C., Crawford M.A., Stuart M.J., Botti J.J. and Demers L.M. (1990). Alterations in transfer and lipid distribution of arachidonic acid in placentas of diabetic pregnancies. Diabetes 39, 914-918.

Lappas M., Permezel M. and Rice G.E. (2004). Release of proinflammatory cytokines and 8-isoprostane from placenta, adipose tissue, and skeletal muscle from normal pregnant women and women with gestational diabetes mellitus. J. Clin. Endocrinol. Metab. 89, 5627-5633.

Lee M.H., Reynisdottir I. and Massague J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev. 9, 639-649.

Loeken M.R. (2005). Current perspectives on the causes of neural tube defects resulting from diabetic pregnancy. Am. J. Med. Genet. C. Semin. Med. Genet. 135, 77- 87.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Mark P.J., Smith J.T. and Waddell B.J. (2006). Placental and fetal growth retardation following partial progesterone withdrawal in rat pregnancy. Placenta. 27, 208-214.

Matsuoka S., Edwards M.C., Bai C., Parker S., Zhang P., Baldini A., Harper J.W. and Elledge S.J. (1995). p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev. 9, 650-662.

Matsuura T., Takahashi K., Nakayama K., Kobayashi T., Choi-Miura N.H., Tomita M. and Kanayama N. (2002). Increased expression of vascular endothelial growth factor in placentas of p57Kip2 null embryos. FEBS Lett. 532, 283-288.

Matuoka S., Thompson J.S., Edwards M.C., Bartletta J.M., Grundy P. and Kalikin L.M., Harper J.W., Elledge S.J., Feinberg A.P. (1996). Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor p57KIP2, on chromosome 11p15. Proc. Natl. Acad. Sci. USA 93, 3026-3030.

Padmanabhan R. and al-Zuhair A.G. (1987). Congenital malformations and intrauterine growth retardation in streptozotocin induced diabetes during gestation in the rat. Reprod. Toxicol. 1, 117-125.

Padmanabhan R. and al-Zuhair A.G. (1990). Ultrastructural studies on the placentae of streptozotocin induced maternal diabetes in the rat. Z. Mikrosk. Anat. Forsch. 104, 212-230.

Padmanabhan R. and Shafiullah M. (2001). Intrauterine growth retardation in experimental diabetes: possible role of the placenta. Arch. Physiol. Biochem. 109, 260-271.

Pedersen J.F. and Molsted-Pedersen L. (1979). Early growth retardation in diabetic pregnancy. Br. Med. J. 1, 18-19.

Polyak K., Kato J.Y., Solomon M.J., Sherr C.J., Massague J., Roberts J.M. and Koff A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor and contact inhibition to cell cycle arrest. Genes Dev. 8, 9-22.

Radaelli T., Varastehpour A., Catalano P. and Hauguel-de Mouzon S. (2003). Gestational diabetes induces placental genes for chronic stress and inflammatory pathways. Diabetes. 52, 2951-2958.

Rao S.S., Chu C. and Kohtz D.S. (1994). Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helixloop-helix regulators. Mol. Cell Biol. 14, 5259-5267.

Robinson J., Canavan J.P., el Haj A.J. and Goldspink D.F. (1988). Maternal diabetes in rats I. Effects on fetal growth and protein turnover. Diabetes 37, 1665-1670.

Shafrir E. and Barash V. (1987). Placental function in maternalfetal fat transport in diabetes. Biol. Neonate 51, 102-112.

Sherr C.J. and Roberts J.M. CDK inhibitors: Positive and negative regulators of G1-phase progression. (1999). Genes Dev. 13, 1501-1512.

Skapek S.X., Rhee J., Spicer D.B. and Lassar A.B. (1995). Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent kinase. Science 267, 1022-1024.

Skapek S.X., Rhee J., Kim P.S., Novitch B.G. and Lassar A.B. (1996). Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. Mol. Cell. Biol. 16, 7043-7053.

Suwaki N., Masuyama H., Masumoto A., Takamoto N. and Hiramatsu Y. (2007). Expression and potential role of peroxisome proliferator-activated receptor γ in the placenta of diabetic pregnancy. Placenta 28, 315-323.

Takahashi K., Nakayama K. and Nakayama K. (2000a). Mice lacking a Cdk inhibitor, p57Kip2, exhibit skeletal abnormalities and growth retardation. J. Biochem. 127, 73-83.

Takahashi K., Kobayashi T. and Kanayama N. (2000b). P57Kip2 regulates the proper development of labyrinthine and spongiotrophoblasts. Mol. Human Reprod. 6, 1019-1025.

Toyoshima H. and Hunter T. (1994). p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell. 78, 67-74.

Yan Y., Frisen J., Lee M.H., Massague J. and Barbacid M. (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. Genes Dev. 11, 973-983.

Yan Y.E., Wang H. and Feng Y.H. (2005). Alterations of placental cytochrome P450 1A1 and P-glycoprotein in tobacco-induced intrauterine growth retardation in rats. Acta Pharmacol. Sin. 26, 1387-1394.

Yokoo T., Toyoshima H., Miura M., Wang Y., Iida K.T., Suzuki H., Sone H., Shimano H., Gotoda T., Nishimori S., Tanaka K. and Yamada N. (2003). p57Kip2 regulates actin dynamics by binding and translocating LIM-kinase 1 to the nucleus. J. Biol. Chem. 278, 52919-52923.

Yu Y., Singh U., Shi W., Konno T., Soares M.J., Geyer R. and Fundele R. (2008). Influence of murine maternal diabetes on placental morphology, gene expression, and function. Arch. Physiol. Biochem. 114, 99-110.

Zhang P., Liegeois N.J., Wong C., Finegold M., Hou H., Thompson J.C., Silverman A., Harper J.W., De Pinho R.A. and Elledge S.J. (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature 387, 151-158.

Zhang P., Wong C., DePinho R.A., Harper J.W. and Elledge S.J. (1998). Cooperation between the Cdk inhibitors p27(KIP1) and p57(KIP2) in the control of tissue growth and development. Genes Dev. 12, 3162-3167.

Accepted July 7, 2011