Histochem Cell Biol (2006) 125: 615–624
DOI 10.1007/s00418-006-0160-y

ORIGINAL PAPER

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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

Accepted: 25 January 2006 / Published online: 21 February 2006
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Abstract Although placental development and implantation depend on the coordination of trophoblast proliferation, differentiation and invasion, little is known about the cell cycle regulators that govern the control of these events. The hypothesis that the coordinated expression of cell cycle progression and inhibition factors will determine whether cytotrophoblasts proliferate or undergo cell cycle arrest or cell cycle exit allowing subsequent differentiation was tested. The cell cycle promotors cyclin A, cyclin B1, PCNA, Ki67 and the cell cycle inhibitors p21, p27 and p57 were immunolocalized in tissue sections of first trimester pregnancies (weeks 6 and 9–12). Double staining with cytokeratin 7 allowed unambiguous identification of extravillous cytotrophoblast (EVT) in the decidua. Villous cytotrophoblasts were immunolabelled for Ki67 and cyclin A but only few were stained with anti-cyclin B1. The syncytiotrophoblast was devoid of immunoreactivity for any of the cell cycle progression factors. It expressed especially p21, whereas p27 and p57 were predominantly found in villous cytotrophoblasts. PCNA, Ki67, cyclin A and cyclin B1 were immunolocalized in proximal and distal EVTs of anchoring villi and in EVT which had invaded the upper decidual segments. All EVTs strongly expressed p27 and p57, but not p21. These data clearly suggest different functions for p21, p27 and p57 in placental development with distinct roles for p21 and p57 in syncytiotrophoblast and EVT differentiation, respectively. p27 appears to be involved in both the processes. The results may also challenge the concept of differential mitotic activity in the proximal and distal parts of the first trimester cytotrophoblast cell column, but more functional studies are clearly needed. The presence of p27 and p57 in EVT cells, which invade the deciduas deeply, may account for the loss of mitogenic potential of these cells.

Keywords PCNA · Ki67 · Cyclin B1 · Cyclin A · CDK inhibitors · Early pregnancy

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Introduction

Human placental development is established by trophoblast invasion into the uterine endometrium and its vasculature. The resulting changes will facilitate an increase in intervillous blood flow and, hence, the exchange of nutrients and molecules between maternal and fetal blood. The transport as well as metabolic and endocrine functions of the placenta reside primarily in the floating villi covered by the syncytiotrophoblast, a tissue that results from terminal differentiation of underlying villous cytotrophoblasts and their subsequent fusion. Anchoring villi establish physical connection of the placenta with the decidua predominantly by a subpopulation of cytotrophoblasts known as extravillous cytotrophoblast (EVT). They accumulate at the tips of the anchoring villi and form cell columns.

Both villous and extravillous cytotrophoblast subpopulations arise by proliferation and differentiation from stem cells located within the cytotrophoblast layer of the chorionic villi (Kaufmann and Castelluci 1997). On the basis of the immunostaining of the Ki67 antigen, a cell cycle regulator with yet unknown role, EVTs have been categorized as the proliferative phenotype, which is primarily located in the proximal part, and the invasive phenotype that is located mainly in the distal part of cell columns (Kaufmann and Castelluci 1997). Current understanding assumes that EVT can differentiate, thereby acquiring an invasive phenotype, which even-

tually enables them to invade the maternal decidua and spiral arteries. Thus placental development involves proliferation and differentiation of the cytotrophoblast in a manner that is tightly regulated in time and space.

The eukaryotic cell cycle is regulated by the coordinated activity of a family of cyclin-dependent kinases (CDKs). These are positively and negatively regulated by the cyclin and CDK inhibitor families (Sherr and Roberts 1995; Xiong 1996). Based on the timing of their appearance in the cell cycle, cyclins can be divided into two groups, i.e. the mitotic cyclins A and B and the G_1 cyclins of the D and E families (Lew et al. 1991). Cyclin A promotes both Gl/S and G2/M transitions, whereas cyclin B1 accumulates in the cytosol during late S phase and G2 and enters the nucleus at the onset of mitosis (Bailly et al. 1992).

In mammalian cells, there are at least two distinct families of CDK inhibitors: the INK4 and the Cip/Kip inhibitors. Both families play regulatory roles during the G_1/S cell cycle checkpoint (Hunter and Pines 1994). Because of their broader panel of CDKs with which they interact (Harper and Elledge 1996), the inhibitors of the Cip/Kip family control other checkpoints as well. p21 plays a role during the G2/M phase transition (Dulic et al. 1998) and may also mediate S phase (Ogryzko et al. 1997) and G2 arrest (Niculescu et al. 1998). Overall it is correlated with cell cycle arrest before terminal differentiation (Parker et al. 1995). Also, p27 has the capacity to arrest cells in G2 (Niculescu et al. 1998). p57Kip2 inhibits cyclin A- and E-associated CDKs and therefore regulates G1/S transition and completion of S phase (Lee et al. 1995) and is primarily expressed in terminally differentiated cells (Yan et al. 1997).

The Ki67 antigen is present only in cells that are within the cell cycle with varying intracellular location depending on the distinct phase of the cell cycle. Its precise role within the network regulating the cell cycle is yet unknown. It is a substrate of the cyclin B/cdc complex and becomes phosphorylated during mitosis (Endl and Gerdes 2000).

PCNA was long regarded only as a proliferation marker because it is expressed late in the G1 phase and early in the S phase (Takahashi and Caviness 1993). Recent evidence demonstrates a much broader range of functions in which PCNA is involved. It is a homotrimer protein that binds to a variety of factors required for cell cycle progression, replication and DNA repair. PCNA relays both positive and negative signals in the cell cycle. It is an essential regulator of p21 activity by binding to the carboxy terminus of p21, which then results in the inhibition of PCNA-dependent DNA replication. In turn p21 controls PCNA levels. When p21 is upregulated during differentiation, PCNA is downregulated. This regulation intimately links PCNA to the cell cycle regulating machinery (Maga and Hubscher 2003). Its expression in cells that lack p21 will allow PCNA to interact with the complexes of cyclin-cyclin-dependent kinases without disruption by p21 and to target the complex to DNA replication proteins.

Despite the importance in understanding the mechanisms controlling proliferation, little is known about how cytotrophoblast proliferation is coordinated with differentiation and what factors determine whether cytotrophoblast cells divide or differentiate and syncytialize. A few studies localized cell cycle regulators that are specifically expressed during key transitions and phases (Ichikawa et al. 1998; Genbacev et al. 2000; DeLoia et al. 1997).

In ongoing studies we aim to shed light on the possible roles of cell cycle related proteins and cell cycle inhibitors during placentation, terminal differentiation, proliferation and invasion of early pregnancy in the humans. Against the background of the novel insights into how PCNA interacts with the cell cycle machinery, we report here in a first step the location of key regulators of the cell cycle PCNA, Ki67, the mitotic cyclins A and B1 as well as the Cip/Kip inhibitors p21, p27 and p57 in first trimester human placenta and decidua samples and relate it to the PCNA expression. The Cip/Kip inhibitor family was chosen because of its broad specificity including not only Cdk4 and Cdk6, but also Cdk1 and Cdk2 and Cdc2. PCNA and Ki67 were used to identify cells in the cell cycle and the mitotic cyclins to label cells with the potential to proliferate, i.e. to go into and through mitosis.

Materials and methods

Tissue collection

Human placental villous and decidual tissue samples were obtained by curettage from legal abortions for social reasons between weeks 6 and 12 post-menstruation. Neither the interrupted pregnancy nor the obstetrical history showed any abnormalities. A total of 18 tissues were analysed: $n=2$ (week 6), $n=8$ (week 9), $n=3$ (week 10), $n=2$ (week 11), $n=3$ (week 12). These samples were supplied by the Department of Obstetrics and Gynaecology at Akdeniz University, Medical Faculty. Written consent was obtained from the women before the procedure. The Ethical Committees of Medical Faculty of Akdeniz University approved the consent forms and protocols to use the tissues.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections of the first trimester tissue. These samples were fixed in 4% formaldehyde for 4 h, embedded and serial sections (6 μm) were collected on SuperFrost Plus slides (Novoglas, Berne, Switzerland). After re-hydrating, samples were transferred to 0.01 M citrate buffer (pH 6) and subsequently heated in a microwave oven for 2×5 min at 750 W for retrieving antigen. After cooling for 20 min at room temperature, the sections were washed with phosphate-buffered saline

(PBS). To remove endogenous peroxidase activity, sections were kept in 3% (v/v) H_2O_2 prepared in methanol for 20 min and subsequently rinsed with PBS for 15 min. Non-specific binding was decreased by normal multi-species serum Ultra V Block (Lab Vision Fremont, USA) at room temperature for 10 min. Sections were incubated overnight or for 60 min at 4°C in a moist chamber with the antibodies listed in Table 1. Labelling was visualized using the Universal LSAB kit (Dako) according to the instructions of the manufacturer. Sections were washed with PBS and stained with 3-amino-9-ethylcarbazole (AEC).

For double labelling, the horseradish peroxidase-labelled streptavidin biotin visualization (LSAB) system was followed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) kit (Dako). The first labelling step used anti-cytokeratin 7 to identify cytotrophoblasts. Staining was developed with diaminobenzidine (DAB) for 3–5 min. After washing with PBS (pH 7.2) the second labelling step followed. To remove endogenous alkaline phosphatase activity, the sections were incubated in levamisole (Dako) for 20 min and washed with PBS for three times, 5 min each. After the washing steps non-specific binding sites were blocked with ultra V block (Lab Vision) for 10 min at room temperature. Following the blocking of non-specific binding, the sections were incubated for 60 min at room temperature in a moist chamber with antibodies against Ki67, PCNA, p27 or p57. Thereafter, the sections were rinsed in PBS and incubated with APAAP system. After rinsing in PBS, alkaline phosphatase was developed with Fast Red for 5–10 min as a chromogene.

All sections were counterstained with Mayers's haemalum (Merck, Darmstadt, Germany) and mounted with Kaiser's glycerol gelatin (Merck). For controls, sections were incubated with non-immune IgG_{2a} (X 0943, Dako), mouse IgG1 (X 0931, Dako), rabbit serum (X 0902, Dako) or rabbit immunoglobulin fraction (X 0903). Pictures were taken with an Axiophot microscope (Zeiss, Oberkochen, Germany).

Two independent observers graded the staining intensity on a semi-quantitative scale ranging from no expression (−), very weak (+/−), moderate (+), strong (++) to very strong (+++) expression.

Immunoblotting

Mononucleated trophoblasts were isolated from first trimester human placental tissue as described (Blaschitz et al. 2000) using enzymatic digestion, subsequent Percoll gradient separation followed by an immunodepletion procedure (Cervar et al. 1999).

Only preparations with a purity ≥99% as assessed by rigorous immunocytochemical characterization (Blaschitz et al. 2000; Cervar et al. 1999) were used to extract proteins by sonication into lysis buffer (50 mM Tris–HCl, pH 7.4, 100 μM EDTA, 100 μM EGTA, 1% NP-40, 0.1% SDS, 0.1% desoxycholic acid) containing protease inhibitor cocktail tablets (Complete; Roche, Nutley, NJ, USA). Total cellular protein (20 μg) was separated by electrophoresis on NuPAGE 4–12% Bis–Tris Gel (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membrane. Immunoblotting for PCNA (1:500), cyclin B1 (1:300), cyclin A (1:300), p21 (1:100), p27 (1:100) and p57 (1:100) used the WesternBreeze system with chemiluminescent detection (Invitrogen). Membranes were exposed to Hyperfilm (Amersham), which was subsequently analysed using an Alpha Digi Doc 1000 gel documentation unit (Alpha Innotech Corporation, CA, USA).

Results

The location of immunohistochemical stainings did not differ between the various gestational weeks included in the study. Therefore, the results summarized in Table 2 do not distinguish between the weeks of gestation. The two observers were always concordant except for the assessments of weak to moderate staining.

Villous tissue

PCNA (Fig. 1a, d), Ki67 (Fig. 1b, e) and cyclin A (Fig. 1c, f) were immunolocalized to villous cytotrophoblasts and stromal cells. In addition, but in contrast to Ki67 (Fig. 1b, e), cyclin A (Fig. 1c, f) and cyclin B1 (Fig. 2b, e), PCNA was also present in the syncytiotrophoblast (Figs. 1a, d, 2a, d). The serial sections immunostained for Ki67, cyclin A and cyclin B1 proteins

Table 1 Antibodies used in this study

Antibodies	Cell cycle phase	Source	Clone	Catalogue no.	Isotype	Dilution[a]
PCNA	G1, S, G2/M	Santa Cruz	PC10	sc-56	Mouse IgG_{2a}	1:1,000
Ki67	G1, S, G2/M	Dako	MIB-1	N1633	Mouse IgG_1	1:1 ready to use
Cyclin A	S, G2	Neomarker	6E6	MS-1061-S	Mouse IgG_1	1:50
Cyclin B1	G2, G2/M	Santa Cruz	GNS1	sc-245	Mouse IgG_1	1:50
p21 WAF-1	G1, S, G2/M	Calbiochem	EA10	OP64	Mouse IgG_1	1:100
p27 Kip1	G1, S, G2	Neomarker		RB-9019-PO	Rabbit serum	1:250
p57 Kip2	G1, S	Santa Cruz		sc-1040	Rabbit IgG	1:250
Cytokeratin7		Dako	OV-TL 12/30	M 7018	Mouse IgG_1	1:750

[a]With Antibody Diluent (Dako, Carpinteria, CA, USA)

Table 2 First trimester staining patterns of cell cycle related proteins at the fetal–maternal interface

	Villous tissue			Extravillous tissue		Decidua			
				Cell column EVT		Cytotrophoblasts		DSCs	DGCs
Cell cycle related proteins	STBs	VCTBs	VSCs	Proximal	Distal	Superficial	Deep		
PCNA	+/−	+ + +	+	+ + +	+ + +	+ + +	+ +	+ +	+ +
Ki67[a]	−	+ + +	+ +	+ +	+ +	+ +	−	+	−
Cyclin A	−	+ + +	+ +	+ +	+ + +	+ +	−	+	−
Cyclin B1[b]	−	−/+	−	+ +	+ +	+	−	+	−
p21	+ + +	−	+/−	−/+	−	−	−	−	−
p27	+	+/−	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+ +
p57	−	+ +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	−

−, no expression; +/−, weak expression; +, moderate expression; + +, strong expression; + + +, very strong expression
STB syncytiotrophoblast, *CTBs* cytotrophoblast cells, *VCTBs* villous cytotrophoblast cells, *VSCs* villous stromal cells, *EVT* extravillous cytotrophoblast, *DSCs* decidual stromal cells, *DGCs* decidual glandular cells
[a]Nuclear staining
[b]Cytoplasmic staining

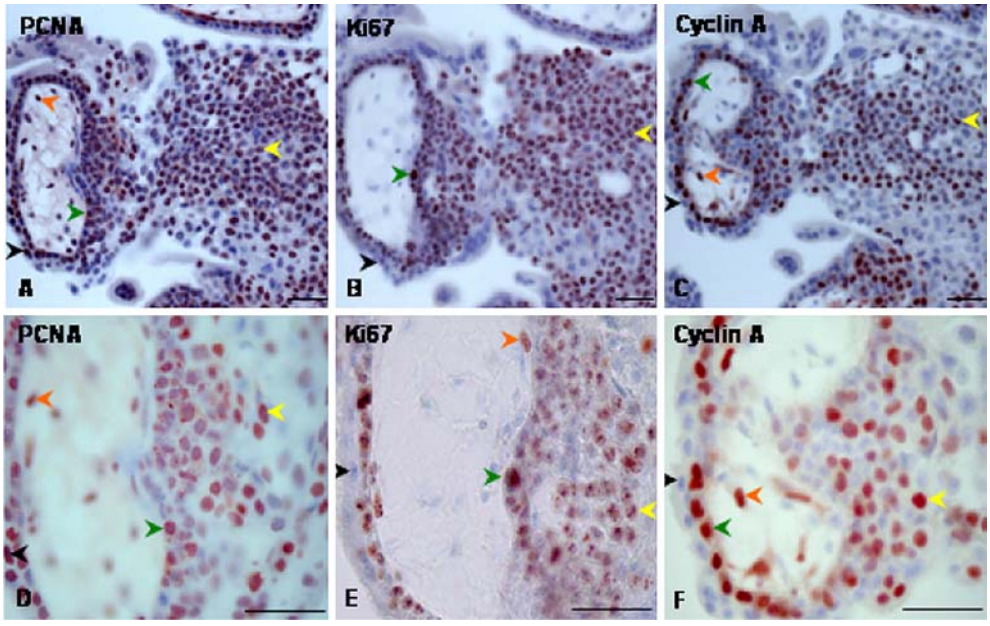


Fig. 1 Immunoreactivity for PCNA, Ki67 and cyclin A in first trimester human placenta (week 6 of pregnancy). **a**, **d** PCNA was present in villous cytotrophoblasts and stromal cells as well as in the proximal and distal EVT cells of cell columns, as well as in few syncytiotrophoblast cells. **b**, **e** Ki67 and **c**, **f** cyclin A were localized in villous cytotrophoblasts and stromal cells as well as in the proximal and distal EVT cells of cell columns. However, fewer EVT cells were stained with cyclin A when compared with Ki67. **d**–**f** are higher magnifications of **a**–**c**, respectively. *Black arrowheads* syncytiotrophoblast, *green arrowheads* cytotrophoblasts, *brown arrowheads* villous stromal cells, *yellow arrowheads* EVT cells in the cell column. *Scale bars*: 50 μm

revealed that the distributions of these proteins were different from PCNA. Interestingly, stromal cells in the villous were not stained with cyclin B1 (Fig. 2b, e).

Immunoreactivity for the cell cycle inhibitor p21 was localized to the syncytiotrophoblast and few stromal cells, but not to the villous cytotrophoblasts (Fig. 2c, f). This contrasted the staining pattern of p27 and p57, both of which were immunolocalized to villous cytotrophoblasts and stromal cells (Fig. 3b, e and c, f, respectively). The syncytiotrophoblast was also labelled for p27 (Fig. 3b, e), but not for p57 (Fig. 3c, f) and cyclin A (Fig. 3a, d) as found in the serial sections. CK7 stained the villous cytotrophoblasts (Fig. 3g–m).

Negative control sections in which the primary antibodies were replaced by their non-immune isotype controls showed no immunoreaction (inserts in Fig. 2a for mouse IgG_{2a}, Fig. 2c for mouse IgG_1).

Extravillous tissue and decidua

CK7 was stained in the cytoplasm of all invading EVT cells in the decidual tissue (Fig. 3g–m). Ki67 immunolabelling was present in both proximal and distal EVT cells of anchoring villi (Figs. 1b, e, 3h, l, 4a) and in EVT cells that had invaded the decidua superficially (Figs. 3h,

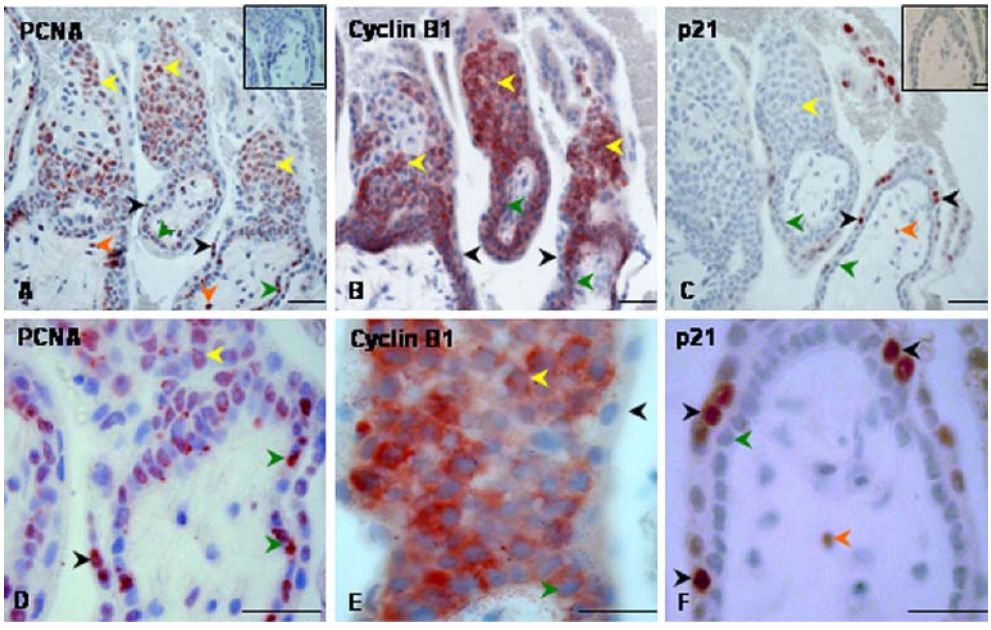


Fig. 2 Immunoreactivity for PCNA, cyclin B1 and p21 in first trimester human placenta (week 9 of pregnancy) at low (**a**–**c**) and high (**d**–**f**) magnification. **a**, **d** PCNA was observed in the syncytiotrophoblast, villous cytotrophoblasts, a few stromal cells and in EVT. **b**, **e** Proximal and distal cells of the cell columns showed cyclin B1 immunoreactivity. However, the syncytiotrophoblast is devoid of staining. **c**, **f** p21 was found in the syncytiotrophoblast and in few villous stromal cells. Control sections show no immunoreactivity (**a**, **c** *inserts*). *Black arrowheads* syncytiotrophoblast, *green arrowheads* cytotrophoblasts, *brown arrowheads* villous stromal cells, *yellow arrowheads* EVT cells in the cell column. *Scale bars*: 50 μm

l, 4a). In contrast, EVT cells that deeply invaded the decidua were not immunoreactive to Ki67 protein (Fig. 3h, l). In general, PCNA immunolabelling paralleled the Ki67 staining pattern in invading EVT cells (Fig. 3j, m). In the serial sections of anchoring villi, immunostaining for Ki67 (Fig. 4a) and the inhibitors, p27 and p57 (Fig. 4b, c, respectively), was similar in the EVT cells that had invaded the superficial decidua.

Immunoreactivity for PCNA (Fig. 5a), Ki67 (Fig. 5b), cyclin A (Fig. 5c), p27 (Fig. 5d) and p57 (Fig. 5e) was found in the decidual stromal cells, but only PCNA and p27 labelling was localized to the glandular epithelium (Fig. 5a, d, respectively).

Cyclin A (Figs. 1c, f, 3a, d) and cyclin B1 (Fig. 6a and insert) were immunolocalized to proximal and distal EVT cells of anchoring villi. Abundant immunostaining for the inhibitors p27 and p57 was seen in the interstitial trophoblast cells, which had deeply invaded the decidua (Fig. 7a, b, respectively). PCNA was also immunostained in EVT cells in deeper decidual segments (Fig. 7c).

Negative controls using non-immune isotype antibodies did not show staining (inserts in Fig. 3b for rabbit IgG and Fig. 4c for rabbit serum)

Specificity of antibodies

Western blotting of total placental tissue extracts did not show any staining because of too low expression levels. However, trophoblasts isolated from first trimester placentas demonstrated specific bands for each protein at the expected molecular weights without significant cross-reactivities (Fig. 8). Ki67 protein has a molecular weight of 345 and 395 kDa. It was not detected within the molecular weight range tested.

Discussion

The present study aimed at identifying the location of mitotic cyclins and cell cycle inhibitors in the first trimester human placenta to improve our understanding of the complex mechanisms governing the control of trophoblast proliferation and differentiation. According to the immunoblots the antibodies used were specific for the target proteins.

Essentially, three original key findings represent the main outcome.

Cip/Kip cell cycle inhibitors

The data clearly suggest different functions for p21, p27, p57 in placental development with distinct roles for p21 in syncytiotrophoblast arrest of mitosis and p27/p57 in cytotrophoblast and stromal cell cycle regulation. The difference between p27 and p57 is the low p27 level in the villous cytotrophoblast which makes its involvement in cell cycle regulation unlikely. When each Cip/Kip inhibitor was knocked out in mice, different phenotypes resulted, thus demonstrating clear differences in biological effects of the Cip/Kip family as a general phenomenon (Nakayama and Nakayama 1998).

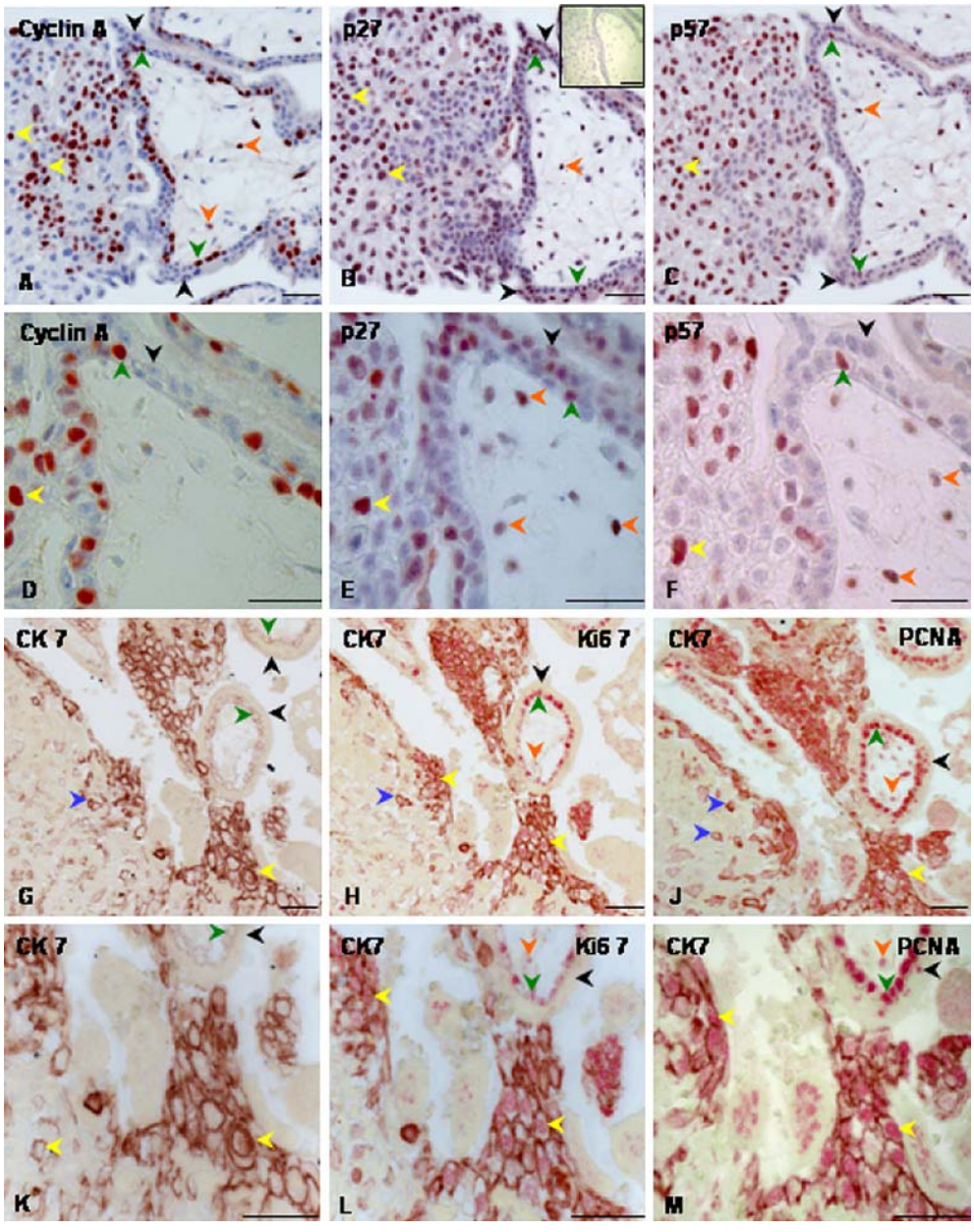


Fig. 3 Immunoreactivity for **a**, **d** cyclin A, **b**, **e** p27 and **c**, **f** p57 was observed in both proximal and distal EVT cells (week 9 of pregnancy). Villous cytotrophoblast and stromal cells were positive for all antibodies but only p27 was positive in syncytiotrophoblasts. Control section shows no immunoreactivity (**b** *insert*). Single and double stainings of proximal and distal EVT cells of the anchoring villi invading the decidua for **g**, **k** CK7, **h**, **l** CK7 and Ki67 and **j**, **m** CK7 and PCNA. **g** CK7 immunolabelled cells represent all cytotrophoblast populations, i.e. villous cytotrophoblast, and EVT in cell columns and decidua. **h** In serial sections and double stainings, Ki67 was localized in both proximal and distal EVT cells of the anchoring villi invading the superficial decidua. **j** PCNA immunostaining was similar to Ki67 protein. Additionally, occasional immunoreactivity was found in the syncytiotrophoblast and in numerous stromal cells. **d**–**f** and **k**–**m** are higher magnifications of **a**–**c** and **g**–**j**, respectively. *Black arrowheads* syncytiotrophoblast, *green arrowheads* cytotrophoblasts, *brown arrowheads* villous stromal cells, *yellow arrowheads* EVT cells in the cell column, *blue arrowheads* only CK7-positive invaded EVT cells. CK7 developed by DAB appears as *brown* whereas Ki67 and PCNA developed by Fast Red appear as *red* colour. *Scale bars*: 50 μm

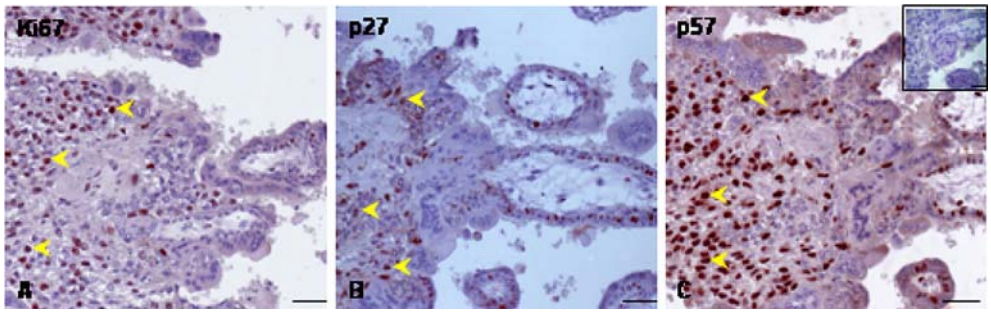


Fig. 4 Immunoreactivity for **a** Ki67, **b** p27 and **c** p57 was observed in both proximal and distal EVT cells of the anchoring villi invading the decidua (week 9 of pregnancy); **c** *insert* control section with no immunoreactivity. *Yellow arrows* EVT cells in the cell column. *Scale bars*: 50 μm

As a hypothesis p57 will inhibit cell cycle progression of the villous cytotrophoblast prior to its fusion with the pre-existing syncytiotrophoblast. Once fusion has occurred, p21 will take over and maintain the syncytiotrophoblast in cell cycle arrest. The presence of cyclin A, but not cyclin B1, in the villous cytotrophoblast along

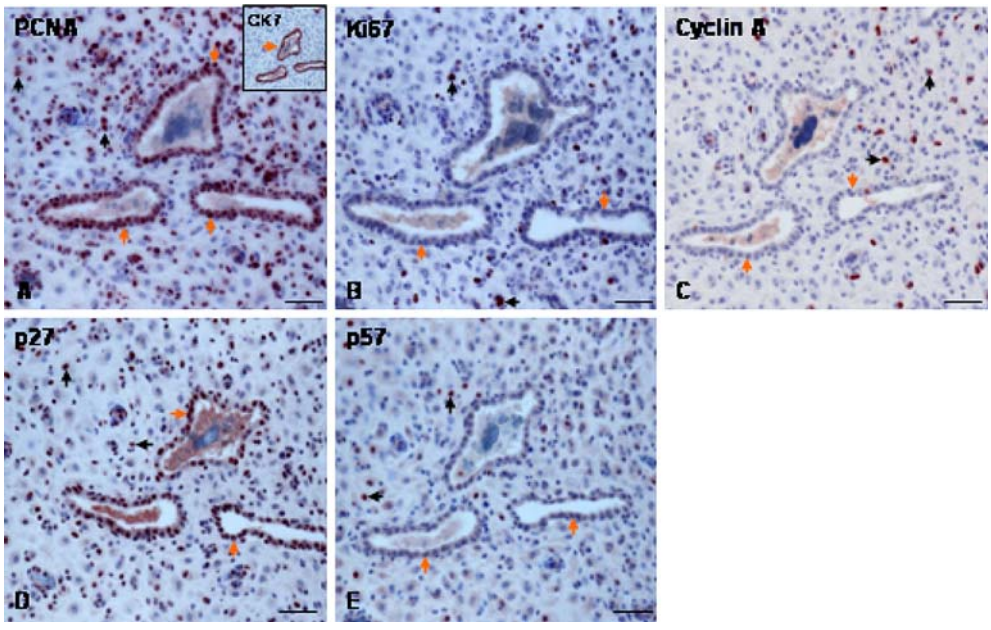


Fig. 5 Immunostaining of PCNA, Ki67, cyclin A, p27 and p57 in decidua (week 11 of pregnancy). **a** PCNA, **b** Ki67, **c** cyclin A, **d** p27 and **e** p57 were present in endometrial stromal cells; however, PCNA and p27 were present in glandular epithelial cells as well. *Insert* in **a** represents CK7 immunostaining. *Brown arrows* glandular epithelial cells, *black arrows* endometrial cell types. *Scale bars*: 50 μm

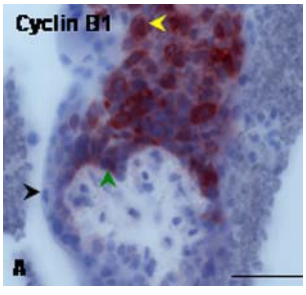


Fig. 6 Immunostaining of cyclin B1 was found in villous cytotrophoblasts and EVT cells of anchoring villi (week 11 of pregnancy). *Insert* shows the anchorage of the villi to decidua with lower magnification. *Black arrow* syncytiotrophoblast, *green arrow* cytotrophoblasts, *yellow arrow* EVT cells in the superficial decidua. *Scale bars*: 50 μm

with the p57 inhibitor expression argues strongly for a cell cycle arrest at the G1/S checkpoint. During or after fusion with pre-existing syncytiotrophoblast the cell cycle is exited as reflected by the complete absence of the mitotic cyclins and Ki67. High p21 levels will serve as additional safeguard to maintain the syncytium in absolute arrest at the G1 phase (Bamberger et al. 1999), should for any reason the syncytium be stimulated to re-enter the cell cycle.

Differentiation of cells induces the expression of P21, which blocks the progression from G1- to S phase. p21 is the only member of the Cip/Kip family that harbours a PCNA binding domain. Therefore, the upregulation of p21 in the syncytiotrophoblast with absent or low levels of PCNA appears to be a characteristic feature of terminal differentiation of the trophoblasts similar to terminally differentiated cardiomyocytes (Engel et al. 2003). Although the weak PCNA immunoreactivity in the syncytiotrophoblast may be the result of active translation, we regard this option as unlikely and rather attribute it to its long half-life and hence to its cytotrophoblast origin with subsequent transfer into the syncytiotrophoblast by fusion.

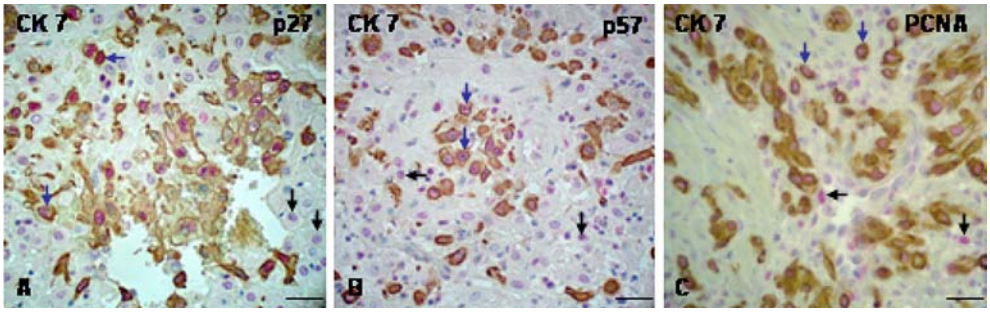


Fig. 7 Double stainings of interstitial EVT cells in the deep parts of deciduas (week 12 of pregnancy): **a** CK7 and p27, **b** CK7 and p57 and **c** CK7 and PCNA. **a** p27, **b** p57 and **c** PCNA were present in EVT cells and other decidual cell types. *Blue arrows* interstitial EVT cells, *black arrows* endometrial cell types. CK7 developed by DAB appears as *brown* colour whereas p27, p57 and PCNA were developed by Fast Red and appear as *red* colour. *Scale bars*: 50 μm

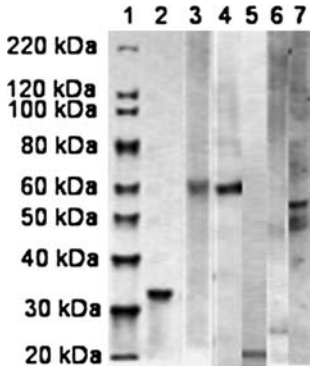


Fig. 8 Representative Western blot of the PCNA, cyclin B1, cyclin A, p21, p27 and p57 proteins in isolated first trimester human trophoblast. *Lane 1* standard, *lane 2* PCNA: 36 kDa, *lane 3* cyclin B1: 60 kDa, *lane 4* cyclin A: 60 kDa, *lane 5* p21: 21 kDa, *lane 6* p27: 27 kDa, *lane 7* p57: 57 kDa

No acute mitotic activity of EVT in cell columns

The proximal and distal EVTs in the cell columns do not differ in their expression pattern of the general cell cycle marker Ki67, the mitotic cyclins A and B1 and the cell cycle inhibitors p27 and p57. This is in contrast to the current opinion (Kaufmann and Castelluci 1997), which is mainly based on the expression pattern of Ki67 (Bulmer et al. 1988). According to that view EVT in the proximal part of the cell column represents the proliferative phenotype and those at the more distal part have exited the cell cycle, lost their proliferative ability and, hence, represent the invasive EVT phenotype. The present data along with evidence that has accumulated over the past years (Genbacev et al. 2000; Ichikawa et al. 1998) strongly challenge this concept. Double immunohistochemical labelling with cytokeratin 7 allowed clear identification of the EVT and their expression of the cell cycle regulators, thus ruling out any confusion with the large leucocyte population present in the decidua at early stages in gestation. Other studies on cell cycle regulators have either not reported on the EVT or not distinguished between proximal and distal cells of the cell columns (Deloia et al. 1997; Bamberger et al. 1999; Olvera et al. 2001; De Falco et al. 2004). The set of cell cycle regulators present in the cell column strongly suggests that the cells in both the proximal and distal parts do not acutely proliferate. However, they have maintained this ability because they have not left the cell cycle, i.e. they express Ki67 and PCNA (this study; Genbacev et al. 2000; Ichikawa et al. 1998) as well as mitotic cyclins. Their mitotic potential is reflected by the phosphorylation of histone H3, which only occurs during mitosis (Genbacev et al. 2000). However, it will require a growth-promoting stimulus of either growth factors or extracellular matrix or both to downregulate the cell cycle inhibitors and allow the cells to fully progress the cell cycle. If proven by independent in vitro methods this will represent a novel mechanism by which the EVTs can respond to the local environment, thus allowing a fine tuning of proliferative activity.

Although these results are straightforward and appear conclusive two additional aspects need consideration.

During the invasion process a proportion of the EVT switches from mitotic division, resulting in diploid EVT, to the endoreduplication cycle leading to tetra-, octa- and even hexadecaploid EVT (Zybina et al. 2002, 2004). The more distal in the cell column and the deeper in the deciduas EVTs are located the higher their ploidy level becomes. Endoreduplication is the result of depletion of mitosis-related proteins associated with p21-induced growth arrest (Chang et al. 2000). Its propagation is accompanied by a loss of cyclin A (Hayashi 1996) and cyclin B1 (MacAuley et al. 1998). The absence of p21 and the high levels of cyclins A and B1 in the EVT across the entire cell column is, therefore, unlikely to be the result of or associated with endoreduplication, but a definitive statement can only be made by experiments addressing the dynamic changes in cell cycle regulators (Hattori et al. 2000). The high cyclin A levels in the EVT are rather the result of the strong p27 expression. p27 is a positive regulator of cyclin D assembly with CDKs, which subsequently hyperphosphorylate the retinoblastoma protein thus leading to activation of E2F-dependent transcription of cyclins E and A (Sherr and Roberts 1999).

The three-dimensional organization of trophoblast cell columns makes possible that two or more columns come in close proximity such that cells may intermingle. Thus, immunoreactivity in a deeper part of a cell column may be due to reactivity with more proximal parts of another column.

Cell cycle exit of EVT in deep deciduas

So far it has been unclear why and how EVT cells lose their proliferative potential when they deeply invade the decidua. Our results strongly argue for a key role of p27 and p57, both strongly expressed in these cells. The presence of these inhibitors will inhibit cell cycle progression and allow the cells to differentiate and change their phenotype. The strong expression of the inhibitors is necessary because cell cycle progression factors are also expressed. They may become active in some situations characterized by a concomitant downregulation of p27 or p57, thus releasing the inhibition and allowing the cells to progress through the cell cycle. Indeed EVT p27 expression is lost in gestational trophoblastic disease such as complete hydatidiform moles and choriocarcinoma (Olvera et al. 2001). In contrast to the cells that have superficially invaded the deciduas, EVT deep in the decidua have lost any proliferative potential similar to the syncytiotrophoblast. Different, however, are the inhibitors that lock the cells in the resting state. These data are at variance from a published report (Genbacev et al. 2000) on the absence of Ki67 in EVT cells that had invaded the superficial parts of decidua. The reason is currently unclear. However, the same study also found

cyclins A and B in this EVT population, thus supporting our conclusion that the cells are in the cell cycle, but arrested at one of the checkpoints.

The concordant p27 and p57 expression in EVT and decidual cytotrophoblasts is not only in line with the strong cooperation between both inhibitors in the control of tissue growth and development but also strongly argues for their key function to keep these cells in cell cycle arrest, especially vis-à-vis the strong expression of the mitotic cyclins. As a hypothesis, any aberrant inhibitor expression, i.e. repression, in EVT and decidual cytotrophoblasts may lead to excessive trophoblast proliferation and invasion such as found in placenta percreta, accreta and increta. However, to the best of our knowledge no studies on cell cycle regulators in these placental pathologies have been made.

Summary

Collectively, the transition from proliferating villous or EVTs to differentiated EVT or syncytiotrophoblast will be made possible by different cell cycle inhibitors. p21 is a specific cell cycle inhibitor for the syncytiotrophoblast that in tandem with PCNA ensures cell cycle arrest. The majority of EVT cells in the proximal and distal parts of cell columns and in the superficial decidua express cell cycle regulators specific for the S and G2 phases of the cell cycle. This is paralleled by high expression levels of p27 and p57. In the absence of p21 because of its downregulation by the strong PCNA expression, these proteins will inhibit cell cycle progression and lead to cell cycle arrest. Therefore, the interstitial and intramural EVT cells cannot proliferate. The results warrant further dynamic studies into the complex mechanisms regulating cell cycle in the various cytotrophoblast populations.

Acknowledgements This study is supported by the Research Fund of Akdeniz University (21.01.0122.02), Antalya, Turkey

References

Bailly E, Pines J, Hunter T, Bornens M (1992) Cytoplasmic accumulation of cyclin B1 in human cells: association with a detergent-resistant compartment and with the centrosome. J Cell Sci 101:529–545

Bamberger A, Sudahl S, Bamberger CM, Schulte HM, Loning T (1999) Expression patterns of the cell-cycle inhibitor p27 and the cell-cycle promoter cyclin E in the human placenta throughout gestation: implications for the control of proliferation. Placenta 20:401–406

Blaschitz A, Weiss U, Dohr G, Desoye G (2000) Antibody reaction patterns in first trimester placenta: implications for trophoblast isolation and purity screening. Placenta 21:733–741

Bulmer JN, Morrison L, Johnson PM (1988) Expression of the proliferation markers Ki67 and transferrin receptor by human trophoblast populations. J Reprod Immunol 14:291–302

Cervar M, Blaschitz A, Dohr G, Desoye G (1999) Paracrine regulation of distinct trophoblast functions in vitro by placental macrophages. Cell Tissue Res 295:297–305

Chang BD, Broude EV, Fang J, Kalinichenko TV, Abdryashitov R, Poole JC, Roninson IB (2000) p21Waf/Cip1/Sdi1-induced growth arrest is associated with depletion of mitosis-control proteins and leads to abnormal mitosis and endoreduplication in recovering cells. Oncogene 19:2165–2170

De Falco M, Fedele V, Cobellis L, Mastrogiacomo A, Giraldi D, Leone S, De Luca L, Laforgia V, De Luca A (2004) Pattern of expression of cyclin D1/CDK4 complex in human placenta during gestation. Cell Tissue Res 317:187–194

DeLoia JA, Burlingame JM, Krasnow JA (1997) Differential expression of G1 cyclins during human placentogenesis. Placenta 18:9–16

Dulic V, Stein GH, Far DF, Reed SI (1998) Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G2/M-phase transition. Mol Cell Biol 18:546–557

Endl E, Gerdes J (2000) The Ki-67 protein: fascinating forms and an unknown function. Exp Cell Res 257:231–237

Engel FB, Hauck L, Boehm M, Nabel EG, Dietz R, von Harsdorf R (2003) p21(CIP1) controls proliferating cell nuclear antigen level in adult cardiomyocytes. Mol Cell Biol 23:555–565

Genbacev O, McMaster MT, Fisher SJ (2000) A repertoire of cell cycle regulators whose expression is coordinated with human cytotrophoblast differentiation. Am J Pathol 157:1337–1351

Harper JW, Elledge SJ (1996) Cdk inhibitors in development and cancer. Curr Opin Genet Dev 6:56–64

Hattori N, Davies TC, Anson-Cartwright L, Cross JC (2000) Periodic expression of the cyclin-dependent kinase inhibitor p57 (Kip2) in trophoblast giant cells defines a G2-like gap phase of the endocycle. Mol Biol Cell 11:1037–1045

Hayashi S (1996) A Cdc2 dependent checkpoint maintains diploidy in Drosophila. Development 122:1051–1058

Hunter T, Pines J (1994) Cyclins and cancer. II Cyclin D and CDK inhibitors come of age. Cell 79:573–582

Ichikawa N, Zhai YL, Shiozawa T, Toki T, Noguchi H, Nikaido T, Fujii S (1998) Immunohistochemical analysis of cell cycle regulatory gene products in normal trophoblast and placental site trophoblastic tumor. Int J Gynecol Pathol 17:235–240

Kaufmann P, Castelluci M (1997) Extravillous trophoblast in the human placenta. Trophoblast Res 10:21–65

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

Lew DJ, Dulic V, Reed SI (1991) Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. Cell 66:1197–1206

MacAuley A, Cross JC, Werb Z (1998) Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. Mol Biol Cell 9:795–807

Maga G, Hubscher U (2003) Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci 116:3051–3060

Nakayama K, Nakayama K (1998) Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. Bioessays 20:1020–1029

Niculescu AB, Chen X, Smeets M, Hengst L, Prives C, Reed SI (1998) Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 18:629–664

Ogryzko VV, Wong P, Howard BH (1997) WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. Mol Cell Biol 17:4877–4882

Olvera M, Harris S, Amezcua CA, McCourty A, Rezk S, Koo C, Felix JC, Brynes RK (2001) Immunohistochemical expression of cell cycle proteins E2F-1, Cdk-2, cyclin E, p27 (kip1), and Ki-67 in normal placenta and gestational trophoblastic disease. Mod Pathol 14:1036–1042

Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW, Elledge SJ (1995) p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. Science 267:1024–1027

Sherr CJ, Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 9:1149–1163

Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1 phase progression. Genes Dev 13:1501–1512

Takahashi T, Caviness VS (1993) PCNA-binding to DNA at the G1/S transition in proliferating cells of the developing cerebral wall. J Neurocytol 22:1096–1102

Xiong Y (1996) Why are there so many CDK inhibitors? Biochim Biophys Acta 1288:1–5

Yan Y, Frisén J, Lee MH, Massagué J, Barbacid M (1997) Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. Genes Dev 11:973–983

Zybina TG, Kaufmann P, Frank HG, Freed J, Kadyrov M, Biesterfeld S (2002) Genome multiplication of extravillous trophoblast cells in human placenta in the course of differentiation and invasion into endometrium and myometrium. I. Dynamics of polyplodization. Tsitologia 44:1058–1067

Zybina TG, Frank HG, Biesterfeld S, Kaufmann P (2004) Genome multiplication of extravillous trophoblast cells in human placenta in the course of differentiation and invasion into endometrium and myometrium. II. Mechanisms of polyplodization. Tsitologia 46:640–648