

# TGF $\beta$ 1 induces multiple independent signals to regulate human trophoblastic differentiation: mechanistic insights<sup>☆</sup>

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## Abstract

Transforming growth factor-beta 1 (TGF  $\beta$ 1) plays a crucial role in controlling trophoblast growth and invasion. Loss of this key regulatory function provides the pathophysiological basis for several tumors, which are characterized by uncontrolled telomerase activity. We have shown earlier that telomerase activity is negatively regulated during terminal differentiation of human trophoblasts, and that TGF  $\beta$ 1 may be an important factor governing the transcription of human telomerase reverse transcriptase (hTERT) (the catalytic subunit of the telomerase complex) during this process. In the present study, we extend these observations to identify possible functional effectors of TGF  $\beta$ 1-induced loss in telomerase activity during human trophoblastic differentiation. We show that this regulation may involve the suppression of c-Myc and an increased production of Mad1. We also observed a simultaneous increase in the expression of cyclin-dependent-kinase inhibitors, p21, p27, p15 and p16, associated with a loss in expression of Cyclin-A2 and Cyclin-E. Thus, TGF  $\beta$ 1 may induce multiple independent signals to check the proliferative potential of human trophoblastic cells and allow their functional differentiation.

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**Keywords:** TGF  $\beta$ 1; Placenta; Trophoblast; Proliferation; Differentiation; Telomerase

**Abbreviations:**  $\beta$ hCG, beta subunit of human chorionic gonadotropin; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction; TGF  $\beta$ 1, transforming growth factor-beta 1.

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## 1. Introduction

The specialized interaction between embryonic and maternal tissues is unique to mammalian development. This interaction begins with the invasion of the uterus by the first differentiated embryonic cells, the trophoblasts, and culminates in formation of the placenta. The human placenta is classified as ‘hemochorial’ because the trophoblasts are in direct contact with maternal blood. Development of this hemochorial placenta depends on the proliferation, migration and invasion of fetal trophoblast cells into the maternal uterus, not unlike locally invasive tumors. However, in contrast to metastatic tumors, trophoblast behavior in situ, is stringently regulated, being confined spatially to the uterus and temporally to early pregnancy (Pijnenborg *et al.*, 1981; Shih and Kurman, 2002). The key to understanding this dramatic turn of events lies in the ‘controlled process of trophoblast differentiation’. Particularly striking is the precision with which these controls function. The importance of intrinsic molecular

controls that effectively circumscribe the extent and duration of trophoblast incursion, becomes increasingly evident in abnormal pregnancies that are characterized by aberrant trophoblast proliferation/invasion (Shih and Kurman, 1997, 2002; Norwitz et al., 2001; Li et al., 2002). The mechanisms that govern this ‘intrinsic control’ are only now beginning to be understood. This process is dependent on a wide variety of molecules produced at the fetomaternal interface. Prime-most among these, are hormones and growth factors. In the present study, we have examined the possible role of one such factor, transforming growth factor-beta 1 (TGF  $\beta$ 1), in controlling human trophoblastic proliferation and differentiation.

TGF  $\beta$ s belong to a family of structurally-related, dimeric, disulphide-linked polypeptides, that includes five TGF  $\beta$  isoforms (TGF  $\beta$ 1–5), activins, inhibins, Mullerian inhibiting substance, Bone morphogenetic proteins, and products of the *Xenopus* Vg1 and *Drosophila* decapentaplegic genes (Godkin and Dore, 1998). In mammals, three isoforms of TGF  $\beta$  (TGF  $\beta$ 1–3) have been identified. These are pluripotent cytokines that play key roles in tumorigenesis, and influence numerous cellular processes. They are known to regulate cell proliferation and differentiation, positively or negatively depending on the cell type, and have been implicated in diverse physiological events such as angiogenesis, expression of the extra-cellular matrix (ECM), immune function, steroidogenesis, and tissue remodeling and repair (Godkin and Dore, 1998). Since all of these events take place during the establishment of pregnancy and during subsequent placental development, it is possible that TGF  $\beta$ s may regulate some of these functions in placental cells as well. Interestingly, TGF  $\beta$  mRNA expression is known to peak at mid-gestation (near 17 weeks) and again, at late gestation (near 34 weeks). The peak expression of TGF  $\beta$  at these stages of placental development is suggestive of its regulation of both trophoblastic invasion and proliferation (Dungy et al., 1991).

Extensive studies have shown that TGF  $\beta$  is an important factor that governs human trophoblast invasion into the maternal uterus (Feinberg et al., 1991; Graham and Lala, 1991; Feinberg and Kliman, 1993; Graham et al., 1994; Irving and Lala, 1995; Feinberg et al., 1995). Interestingly, TGF  $\beta$  is the prototypical micro-environmental regulatory molecule that signals cell cycle arrest, primarily in epithelial cells, by counteracting the effects of many different mitogenic growth factors (Roberts and Sporn, 1993). Many actively dividing cell-types show an anti-proliferative response to TGF  $\beta$ 1, where it is proposed to act as a tumor suppressor (Knabbe et al., 1987; Massague, 1990; Katakura et al., 1999). However, not much is known about how TGF  $\beta$  acts to control human trophoblast proliferation.

We have shown earlier that human trophoblast differentiation is accompanied by increased TGF  $\beta$ 1 expression (Rama et al., 2001). These results pointed to a role for TGF  $\beta$ 1 in the differentiation process per se. As expected, human trophoblastic cells were found to differentiate into functionally mature syncytiotrophoblasts following TGF  $\beta$ 1-treatment. We also demonstrated that TGF  $\beta$ 1 could directly modulate trophoblast telomerase activity by specifically suppressing human telomerase reverse transcriptase (hTERT; the catalytic subunit of the telomerase holo-enzyme complex) gene expression. Several lines of evidence indicate that hTERT expression is crucial for telomerase activity, and that it constitutes a key step in the development of human cancers (see Rama et al., 2001). Given this, the loss in hTERT expression that we observe during human trophoblastic differentiation is of considerable significance. TGF  $\beta$ 1 could thus be a crucial factor that serves to keep the proliferative potential of these ‘tumor-like cells’ in tight check. In the present report, we extend these observations to describe possible functional effectors of TGF  $\beta$ 1-induced trophoblastic differentiation, a process that also involves modulation in telomerase activity.

TGF  $\beta$ 1 is known to classically mediate its anti-proliferative/anti-mitogenic activities in epithelial cells, through two classes of rapid gene responses (Moses et al., 1990; Geng and Weinberg, 1993; Raynal et al., 1997):

- 1) Down-regulation of c-Myc
- 2) Signals that directly compromise the expression/function of cyclins and cyclin-dependent-kinase (Cdk) inhibitors.

While the effects of TGF  $\beta$ -signaling on muscle, bone and cartilage cell differentiation are relatively well studied, little is known about how TGF  $\beta$  brings about human trophoblastic differentiation. Also, the regulation of many of these factors known to affect trophoblast function remains obscure. Consequently, we have examined the modulation of some important cell cycle-regulatory proteins, during TGF  $\beta$ 1-induced trophoblastic differentiation.

The proto-oncogene c-Myc, is a member of a transcriptional regulatory network, responsible for controlling a set of genes whose functions impinge directly on the machinery of cell growth and proliferation (reviewed in Grandori et al., 2000). Since the c-myc gene is actively transcribed in many proliferating cells, and alteration of c-myc expression is associated with a large number of tumors of diverse origin, it was of interest to study the expression of c-myc during human trophoblastic development. Very few studies have examined the mediation of trophoblastic differentiation events by proto-oncogenes, especially following growth factor stimulus.

We next analyzed the expression of a variety of cyclins (Cyclins A2 and E) and cyclin-dependent-kinase (Cdk)-inhibitors (p16<sup>Ink4a</sup>, p15<sup>Ink4b</sup>, p21<sup>Cip1</sup> and p27<sup>Kip1</sup>) during TGF  $\beta$ 1-induced trophoblastic differentiation. This study constitutes an important aspect of placental growth because it is these molecules that essentially govern cell cycle progression. It is critical that a cell stops proliferating and exits the cell cycle, so that it can now respond to signals that would permit differentiation.

We have also analyzed the expression of the Myc/Mad/Max transcriptional module during human trophoblastic differentiation. This network encompasses a group of helix–loop–helix/leucine zipper transcription factors. Specifically, c-Myc or Mad 1 associates with the ubiquitously produced Max protein to form a heterodimer capable of either gene activation or repression, respectively (Gunes et al., 2000). As recalled earlier, c-Myc drives progression of cells through the cell cycle, and its expression is highest in actively proliferating cells. On the other hand, Mad 1 gene expression is predominantly associated with differentiating compartments in many organs and across a broad spectrum of cell types (McArthur et al., 1998). Max is an obligate partner for both Myc and Mad function. The strikingly distinct patterns of expression of these transcription factors in many cell types, prompted us to investigate a possible role for this transcriptional network in human trophoblastic growth and differentiation.

## 2. Materials and methods

### 2.1. Collection of human placental tissue

Human placentae obtained immediately following spontaneous vaginal deliveries or uncomplicated cesarean sections at term, were collected in sterile, ice-cold phosphate-buffered saline (PBS), pH 7.4, containing 200 U/ml Penicillin, 100  $\mu$ g/ml Gentamycin and 25 U/ml Nystatin. The tissue was washed extensively in ice-cold PBS before processing, to get rid of blood contamination. All antibiotics were purchased from Hi-Media, India. Collection of human placental tissue was performed with the consent of the patients concerned and under the guidelines set up by the Institute's Ethics Committee.

### 2.2. Isolation of human trophoblasts

Cytotrophoblasts were isolated from normal human term placentae according to the protocol described by Kliman et al. (1986), with minor modifications as detailed in Rama et al. (2001). The purity of the cell preparation (determined by immunostaining for Cytokeratin and absence of staining for Vimentin) was

greater than 97%. Terminally differentiated syncytiotrophoblasts were isolated from human term placentae following the procedure described by Kaspi and Nebel (1974), with minor modifications as detailed in Rama et al. (2001). The isolated multinucleate cells were found to be negative for telomerase activity (Rama et al., 2001) and positive for  $\beta$ -subunit of human chorionic gonadotropin ( $\beta$ -hCG), a marker for the terminally differentiated syncytiotrophoblast.

### 2.3. Cell culture and induction of differentiation using TGF $\beta$ 1 in human trophoblastic cells

BeWo cells (a human choriocarcinoma cell line; generously gifted by Dr. Susan Fisher, University of California, San Francisco, CA) were cultured in DMEM (Dulbecco's modified Eagles medium)/Ham's F-12 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) and antibiotics, at 37 °C, in an atmosphere of 5% CO<sub>2</sub>. A total of 0.5–1  $\times$  10<sup>6</sup> cells/ml per well were plated in 6 well multi-dishes and maintained in culture until the cells were 70–80% confluent at which time, they were harvested for protein or RNA (0-h samples). Differentiation was induced in these cells by the addition of 10 ng/ml recombinant human TGF  $\beta$ 1 (gifted by Professor P. Kondaiah, Department of MRDG, IISc) (Rama et al., 2001). For experiments involving induction of differentiation, cells were maintained in serum-free medium (as described by Taylor et al., 1991, with minor modifications) prepared with DMEM/Ham's F-12 medium supplemented with insulin (1  $\mu$ g/ml), transferrin (5  $\mu$ g/ml), Bovine Serum Albumin (500  $\mu$ g/ml) and antibiotics. Following treatment, cells were harvested at specific time-points for protein or RNA. All experiments involving treatment of cells with TGF  $\beta$ 1 were accompanied in parallel by vehicle-controls (HB-treated cells; HB: 4mM HCl+50  $\mu$ g/ml BSA; Rama et al., 2001). Treatment of cells with HB induced NEITHER morphological (formation of multinucleate syncytia), nor biochemical (increase in  $\beta$ -hCG production) changes associated with trophoblast differentiation (data not shown in the present manuscript, please refer to Rama et al., 2001). More importantly, treatment of trophoblastic cells with HB did not alter their telomerase activity (an important parameter to judge replicative potential) (Rama et al., 2001), thereby validating this experimental system for detection of authentic TGF  $\beta$ 1-induced effects.

The viability and number of cells following treatment was verified using the MTT assay (Mosmann, 1983). All fine chemicals used for these experiments were purchased from Sigma, St. Louis, MO, unless otherwise mentioned.

#### 2.4. Semi-quantitative RT-PCR

Total RNA was extracted from isolated trophoblasts or from treated BeWo cells, at given time-points, using guanidium thiocyanate as described by Chomczynski and Sacchi (1987). The integrity of the isolated RNA was checked on a 1% MOPS-HCHO agarose gel and the quantity of RNA estimated spectrophotometrically. A total of 2 µg of total RNA was treated with 2 U DNase I (Stratagene, La Jolla, CA) along with 15 U RNase inhibitor (Roche Molecular Biochemicals, Germany) for 1 h at 37 °C. The enzyme was denatured at 65 °C for 15 min and the RNA precipitated using 3 volumes of ethanol and 1/10th volume of 3 M sodium acetate, pH 5.2. Reverse transcription was carried out using 1.5 µM random hexamers (Roche Molecular Biochemicals, Germany) and 100 U MMLV-RT (Promega Corporation, Wisconsin, MI) in a 20 µl reaction mixture (containing 1 × RT buffer and 500 µM dNTPs). Following a 1-h incubation at 37 °C, the enzyme was inactivated by heating at 95 °C for 5 min. A 2-µl aliquot of this solution was then used for PCR amplification using specific primers. A simultaneous control without reverse transcriptase ('Minus RT' negative-control) was included for every reaction, to verify absence of non-specific amplification that could result from residual genomic DNA contamination.

cDNA amplifications used highly specific forward and reverse primers with an initial heating at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, annealing temperature for 1 min and 72 °C for 2 min, on a PCR Thermal Cycler (MJ Research, USA). PCR details are compiled in Table 1. A 12-µl aliquot of the PCR products was electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide

(EtBr). Each figure is representative of three independent experiments.

Radioactive RT-PCR was performed to detect p21<sup>Cip1</sup> transcripts. The protocol followed was essentially the same as that described above, except for the following modifications:

- 1) 0.2 µCi of  $\alpha$ [<sup>32</sup>P]dCTP (NEN Life Science Products, MA, USA; Specific activity of label used: 3000 Ci/mmol) was included in every PCR reaction.
- 2) The number of cycles in PCR was reduced to 24.
- 3) 6 µl of the PCR-reaction-mixture was resolved on a 1.5% agarose gel. After the run was complete, the gel was dried onto Whatman 3M paper and exposed to an X-Ray film (Indu, Hindustan Photo films, India) for 5–6 h at –70 °C. Signals were visualized by developing the film. Each figure is representative of three independent experiments.

Signal intensities of various bands were determined using the Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120). The expression levels of specific transcripts were inferred upon normalizing their signal intensities to that of glyceraldehyde 3-phosphate-dehydrogenase (GAPDH), which served as an internal control in this semi-quantitative analysis. Values so obtained were used to determine Mean ± S.E. for a graphical representation. 'Significance or *P*-value' was determined using standard statistical tests as described elsewhere in this section.

The authenticity of all the RT-PCR products described in this study, was confirmed by sequencing, following their purification from low-melting agarose gels using the commercially available GFX<sup>TM</sup> PCR DNA/Gel Band Purification kit (Amersham Pharmacia Biotech., UK). Sequencing was performed at the DNA

Table 1  
PCR details

| Product             | Primers  | Product size (bp) | PCR annealing temperature (°C) | References (if any)   |
|---------------------|--|-------------------|--------------------------------|-----------------------|
| GAPDH               | F-5' GGAGTCAACGGATTTGGT 3'<br>R-5' GTGATGGGATTTCATTGAT 3'    | 206               | 60                             | Wolfahrt et al., 1998 |
| p27 <sup>Kip1</sup> | F-5' AGGATGTACGCGGAGCCGC 3'<br>R-5' CTTCTTGGGCGTCTGCTCCA 3'  | 251               | 60                             | Robson et al., 1999   |
| p21 <sup>Cip1</sup> | F-5' CTCAGAGGAGGCGCCATG 3'<br>R-5' GGGCGGATTAGGGCTTCC 3'     | 517               | 54                             | Robson et al., 1999   |
| c-myc               | F-5' AAGTCCTGCGCCTCGCAA 3'<br>R-5' GCTGTGGCCTCCAGCAGA 3'     | 249               | 54                             | Wang et al., 2000     |
| Mad 1               | F-5' TTCAGCGAGAGCAGCGACAC 3'<br>R-5' CAGAACCAACAGGGAGAACC 3' | 352               | 55                             | Willey et al., 1998   |
| Max                 | F-5' TGACATCGAGGTGGAGAGCG 3'<br>R-5' CCCCCATCGAAGGCAGAAAT 3' | 396               | 58                             | Willey et al., 1998   |
| Cyclin A2*          | F-5' CGTGGACTGGTTAGTTGA 3'<br>R-5' ATGGCAAATACTTGAGGT 3'     | 416               | 54                             | Present study         |

All other primers were synthesized by Microsynth GmbH, Switzerland.

\* Primers synthesized by Bangalore Genei, India.



Sequencing Facility, IISc, using the ABI Prism 377 automated DNA sequencer.

### 2.5. Preparation of protein lysates

Cell cultures were washed thrice with PBS (pH 7.4) at given time-points and treated with ice-cold lysis buffer (50 mM Tris, pH 8.0; 150 mM sodium chloride; 0.1% SDS; 0.02% sodium azide; 1% Nonidet-P40 and 0.5% sodium deoxycholate) containing 100 µg/ml PMSF and 1 µg/ml Aprotinin, for 20 min on ice. Protein lysates were clarified by centrifugation at  $15\,000 \times g$  for 20 min at 4 °C, and protein content was determined by the Bradford's assay (Bradford, 1976). Aliquots of protein lysate were stored at –70 °C until analyzed. All chemicals used for lysis were purchased from Sigma, St. Louis, MO.

### 2.6. Western Blot analysis

Equal quantities of protein (100 µg) were electrophoresed on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose (Sartorius AG, Germany) membranes, using a semi-dry transfer apparatus. After completion of transfer, the membranes were blocked in 5% non-fat milk (Nestle, India) in tris-buffered-saline (TBS) and incubated overnight at 4 °C with the corresponding dilution of the primary antibody. Membranes were washed several times with TBS containing 0.05% Tween-20 (TBST) and incubated with the appropriate horse-radish-peroxidase (HRP)-conjugated secondary antibody for 1 h. Following extensive washing in TBST, bound antibodies were detected by an enhanced chemi-luminescence (ECL) detection system using the protocol recommended by the manufacturers (Amersham-Pharmacia Biotech., UK). Details of antibodies used for Western Blot analyses are compiled in Table 2. Following hybridization, the blot was stripped and re-probed (again, using the protocol recommended by the manufacturers) for Actin, which served as an internal control for assessing equality of protein loading. Signal intensities of various bands were determined using the

Kodak Electrophoresis and Gel Documentation Analysis System (EDAS-120).

The expression levels of specific protein products were inferred upon normalizing their signal intensities to that of Actin (for quantitation of Cyclin E blots, signal intensities corresponding to both isoforms were added to provide one value). Values so obtained were used to determine Mean  $\pm$  S.E for a graphical representation. Significance or 'P-value' was determined using standard statistical tests, as described below. Each figure is representative of three independent experiments (independent cultures).

### 2.7. Statistical analysis

Data are represented as Mean  $\pm$  S.E. of at least three separate experiments performed with the same treatment protocol. For statistical comparison among groups involving only two columns (RT-PCRs in cytotrophoblasts vs syncytiotrophoblasts), significance was evaluated using the unpaired two-tailed *t*-test. For comparison of data comprising three or more columns (RT-PCRs or Western blot analyses involving three or more time-points), statistical significance was evaluated using the Kruskal–Wallis non-parametric one-way ANOVA test. In both instances, a 'P-value' less than or equal to 0.05 was considered to be statistically significant. For experiments with multiple time-points, the *P*-value indicated in the figures, represents the significant difference between the star-marked column mean and the corresponding 0-h/untreated control.

## 3. Results

### 3.1. Regulation of *c-myc* expression by TGF $\beta$ 1 in differentiating trophoblastic cells

BeWo cells were cultured in the presence of 10 ng/ml TGF  $\beta$ 1 for a period of 72–96 h. During this course of time, differentiating trophoblastic cells were harvested at regular intervals and analyzed for the expression of *c-myc* mRNA and protein. RT-PCR analysis indicates a

Table 2  
List of antibodies used for Western blot analyses

| Primary antibody     | Antibody type     | Dilution used (1° Ab) | Secondary antibody  | Dilution used (2° Ab) |
|----------------------|-------------------|-----------------------|---------------------|-----------------------|
| Actin                | Monoclonal        | 1:250                 | Anti-mouse IgG-HRP  | 1:6000                |
| p27 <sup>Kip1</sup>  | Rabbit polyclonal | 1:250                 | Anti-rabbit IgG-HRP | 1:3000                |
| p21 <sup>Cip1</sup>  | Rabbit polyclonal | 1:200                 | Anti-rabbit IgG-HRP | 1:3000                |
| p16 <sup>Ink4a</sup> | Monoclonal        | 1:200                 | Anti-mouse IgG-HRP  | 1:6000                |
| p15 <sup>Ink4b</sup> | Rabbit polyclonal | 1:200                 | Anti-rabbit IgG-HRP | 1:3000                |
| p-c-Myc              | Rabbit polyclonal | 1:300                 | Anti-rabbit IgG-HRP | 1:3000                |
| Cyclin E             | Rabbit polyclonal | 1:200                 | Anti-rabbit IgG-HRP | 1:3000                |

All antibodies were procured from Santa Cruz Biotechnology Inc., CA, USA.

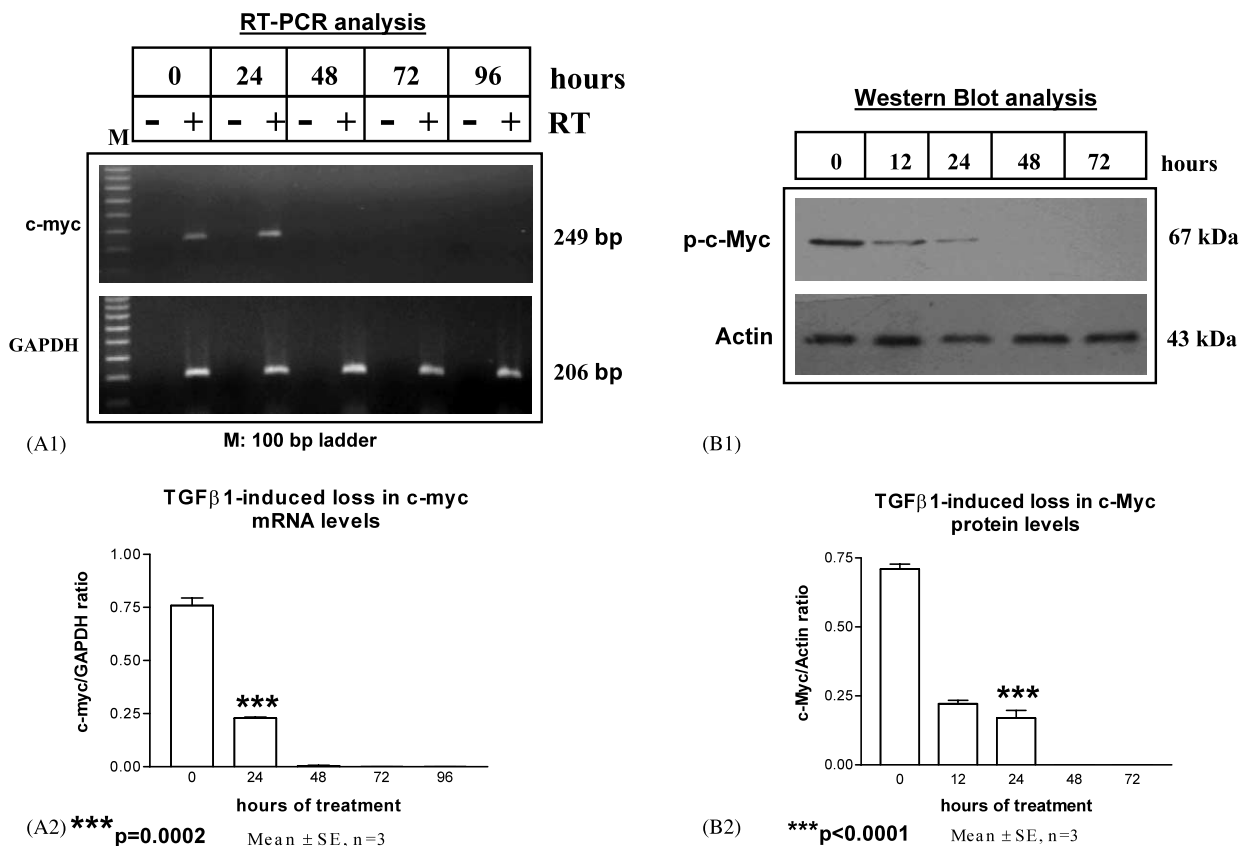


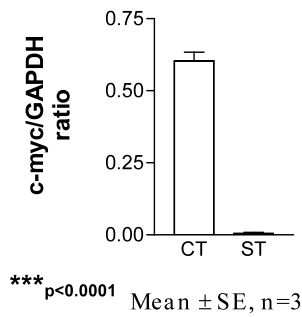
Fig. 1. Regulation of c-Myc expression by TGF  $\beta$ 1 in differentiating BeWo cells.  $1 \times 10^5$  cells/well were plated in a 24-well culture plate and treated with 10 ng/ml rhTGF  $\beta$ 1 in serum-free medium supplemented with 1  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin and 500  $\mu$ g/ml BSA. Cultures were harvested at regular intervals for RT-PCR and Western Blot analyses. (A1) RT-PCR for c-myc. RNA isolated from 0-h sample, and from differentiating BeWo cells at 24, 48, 72 and 96 h following TGF  $\beta$ 1-treatment were reverse-transcribed, and the cDNA so obtained was subjected to semi-quantitative PCR in the linear range of amplification, with GAPDH amplification as an internal control. Panel A2 is a graphical representation of this data. Values represent Mean  $\pm$  S.E. from three independent experiments. (B1) Western Blot analysis for c-Myc. BeWo cells at 0-h, and after 12, 24, 48 and 72 h following TGF  $\beta$ 1-treatment, were lysed and 100  $\mu$ g of protein from each sample was electrophoresed on 10% SDS-PAG, transferred onto a Nitrocellulose membrane, and probed with antibody to p-c-Myc (phosphorylated form of c-Myc). The blot was stripped and re-probed for Actin, which served as an internal control. Panel B2 is a graphical representation of this data. Values represent Mean  $\pm$  S.E. from three independent experiments.

substantial loss in expression of c-myc transcripts following 24 h of TGF  $\beta$ 1-treatment (Fig. 1A1). A2 is a graphical representation of this data. This significant loss in c-myc mRNA levels was also paralleled by a corresponding, time-dependent loss in c-Myc protein levels, as judged by Western blot analysis (Fig. 1B1, B2). We could not detect the c-Myc protein in differentiating trophoblastic cells after 24 h of TGF  $\beta$ 1-treatment.

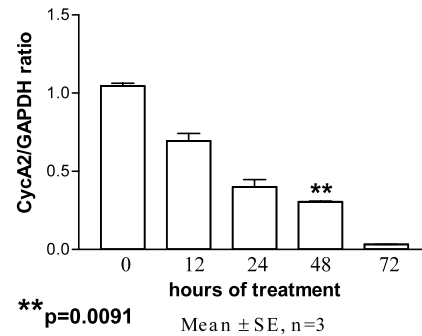
In order to assess if these observations truly reflect the differentiation of human trophoblasts *in vivo*, we performed similar experiments on cytotrophoblasts and syncytiotrophoblasts directly isolated from human term placentae (instead of allowing the terminally differentiated cells to form over a period of time in culture). RT-PCR analysis revealed that c-myc transcripts were restricted to the proliferative mononuclear cells (Fig. 2A). We could not detect the c-myc message in the terminally differentiated syncytiotrophoblast sample.

### 3.2. Regulation of cyclins A2 and E by TGF $\beta$ 1 in differentiating trophoblastic cells

Human trophoblastic BeWo cells were allowed to differentiate in the presence of 10 ng/ml TGF  $\beta$ 1, for a period of 72–96 h. Cells were harvested at regular intervals and analyzed for the expression of cyclins A2 and E. RT-PCR analysis indicates a significant loss in expression of cyclin A2 transcripts with onset of terminal differentiation (Fig. 2B). The loss was substantial following 48 h of TGF  $\beta$ 1-treatment. Western blot analysis for cyclin E also reveals a similar pattern. We observed a significant loss in expression of cyclin E protein during the differentiation process (Fig. 3A, B). There are two known isoforms of cyclin E, sized 52- and 50 kDa (McKenzie et al., 1998) and the antibody that we have used (obtained from Santa Cruz Biotech., USA), recognizes both these isoforms. Hence, we observe a doublet signal. Human trophoblastic cells were found to

**c-myc mRNA levels in CT and ST****CT : Cytotrophoblast sample****ST : Syncytiotrophoblast sample**

(A)

**TGFβ1-induced loss in CycA2 mRNA levels**

(B)

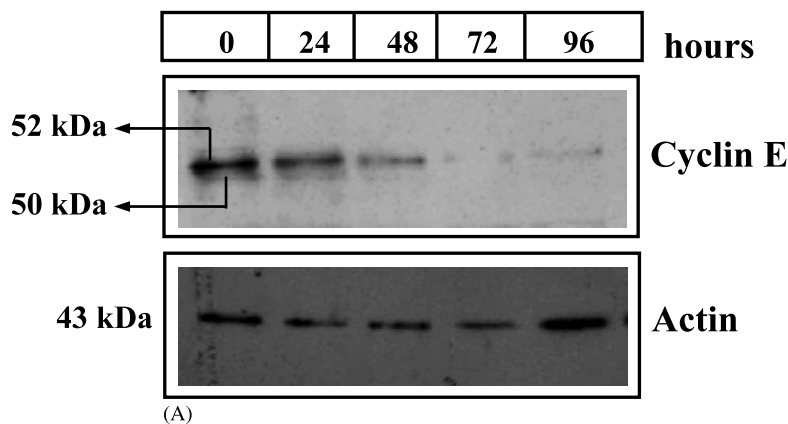
Fig. 2. Regulation of c-myc and cyclin-A2 expression during human trophoblast differentiation. (A) RT-PCR for c-myc in human trophoblasts. Cytotrophoblasts (CT) and syncytiotrophoblasts (ST) were isolated separately from human term placentae and processed for RT-PCR analysis for c-myc, with GAPDH amplification as an internal control. Values represent Mean ± S.E. from three independent experiments. (B) RT-PCR for cyclin A2 in differentiating BeWo cells. RNA isolated from 0-h sample, and from differentiating BeWo cells at 12, 24, 48 and 72 h following TGF β1-treatment were subjected to semi-quantitative RT-PCR for cyclin A2, with GAPDH amplification as an internal control. Values represent Mean ± S.E. from three independent experiments.

predominantly express the larger isoform (i.e. the 52 kDa protein). The expression of cyclins A2 and E, was almost totally undetectable following 48 h of TGF β1-treatment.

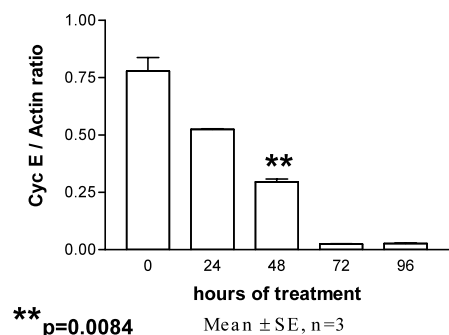
### 3.3. Regulation of cyclin-dependent-kinase (Cdk) inhibitors by TGF β1 in differentiating trophoblastic cells

Differentiating BeWo cells were harvested at regular intervals following treatment with 10 ng/ml TGF β1, and analyzed for the expression of various Cdk inhibitors. Western blot analyses for p16<sup>Ink4a</sup> and p15<sup>Ink4b</sup> indicate increased production of these Cdk inhibitors

during the differentiation process (Fig. 4A, B). We could not detect the presence of these proteins in 0-h/untreated cells. p15 and p16 levels were highest at 48 h, following which, there was a slight decrease. RT-PCR analysis for p27<sup>Kip1</sup> in differentiating BeWo cells, clearly reveals increased expression of this Cdk inhibitor in syncytiotrophoblasts that formed with time after TGF β1-treatment (Fig. 5A1). This significant increase in p27 mRNA levels was also paralleled by an increase in p27 protein levels, as judged by Western blot analysis (Fig. 5A2). Actively proliferating 0-h/untreated cells also expressed this protein (at lower levels, though, when compared to fully differentiated cells). We also observed

**Western Blot analysis**

(A)

**TGFβ1-induced loss in Cyc E protein levels**

(B)

Fig. 3. Regulation of cyclin E expression by TGF β1 in differentiating BeWo cells. (A) Western Blot analysis for cyclin E. BeWo cells at 0-h, and after 24, 48, 72 and 96 h following TGF β1-treatment, were harvested and analyzed for the expression of cyclin E protein. The blot was stripped and re-probed for actin, which served as an internal control. Panel B is a graphical representation of this data. Values represent Mean ± S.E. from three independent experiments.

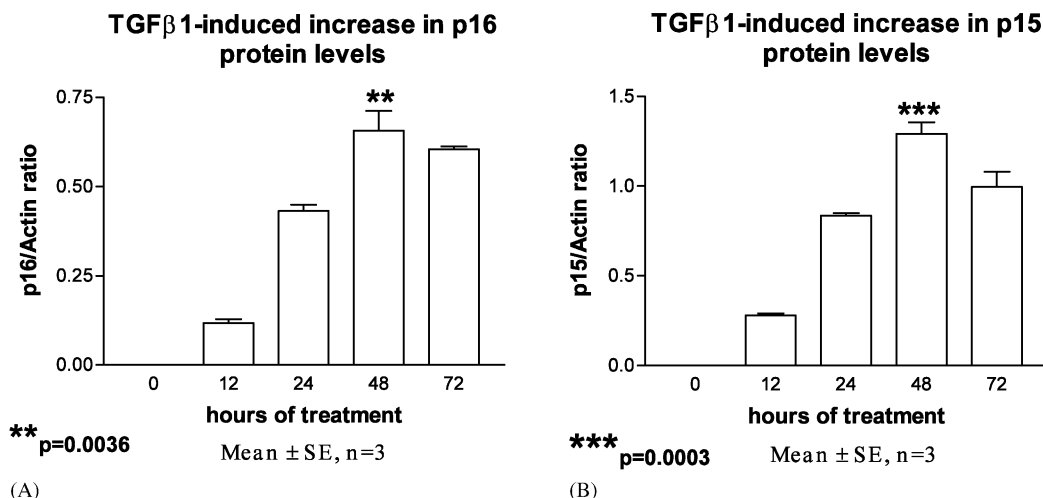


Fig. 4. Regulation of p16<sup>Ink4a</sup> and p15<sup>Ink4b</sup> expression by TGF β1 in differentiating BeWo cells. BeWo cells at 0-h, and after 12, 24, 48 and 72 h following TGF β1-treatment, were harvested and analyzed for the expression of p16 (Panel A) and p15 protein (Panel B) by Western blot analyses. The blots were stripped and re-probed for actin, which served as an internal control. Values represent Mean ± S.E. from three independent experiments.

a similar increase in the expression of p21<sup>Cip1</sup> in differentiating cells, both by RT-PCR (Fig. 5B1), and by Western blot analysis (Fig. 5B2). However, we could not detect the presence of p21 in 0-h/untreated cells.

### 3.4. Regulation of the Myc/Mad/Max module during human trophoblastic differentiation

BeWo cells were allowed to differentiate in the presence of 10 ng/ml TGF β1 for 96 h in culture. Cells were harvested at regular intervals and analyzed for the expression of c-Myc, Mad 1 and Max mRNA by RT-PCR. Results reveal a substantial loss in expression of c-Myc transcripts with onset of trophoblastic differentiation (Fig. 6A; already described in Fig. 1). We could not detect c-Myc mRNA in differentiating BeWo cells after 24 h of TGF β1-treatment. This was accompanied in parallel by a significant increase in the expression of Mad 1 (Fig. 6B). Mad 1 transcripts were undetectable in 0-h/untreated BeWo cells, but increased soon after TGF β1-treatment. Max mRNA levels also tended to a slight increase with differentiation (Fig. 6C), although the changes were far less dramatic than in the case of either c-Myc or Mad 1 mRNA.

## 4. Discussion

The human placenta is a dynamic organ that undergoes remarkable periodic growth, remodeling and breakdown. In normal pregnancies, the villous trophoblast of the placenta consists of a population of proliferating cytotrophoblasts that eventually differentiate and individually fuse to form multinucleate syncytiotrophoblasts. In spontaneous abortions, the

trophoblasts are less hyperplastic (van Lijnschoten et al., 1994), and degenerative lesions are found in the placental tissue (Benirschke and Kaufmann, 2000). On the other hand, gestational trophoblastic tumors are characterized by the presence of highly proliferative neoplastic trophoblasts (Kohorn et al., 2000; Chen et al., 2002). Therefore, trophoblasts from placentae of normal pregnancies, from abortions and intra-uterine growth retardation (IUGR) placentae, and from cases of gestational trophoblastic tumors, seem to differ in terms of their respective rates of proliferation.

Throughout human gestation, the fetomaternal interface is rich in biologically active TGF β (Chakraborty et al., 2002). Interestingly, TGF β1 has also been shown to significantly inhibit the fetal bovine serum (FBS)-stimulated proliferation of Rcho-1 rat choriocarcinoma cells (Hamlin and Soares, 1995). Considering the central role of TGF β1 in tumorigenesis, its importance in the induction of human trophoblast differentiation, and its ability to directly modulate trophoblast telomerase activity (Rama et al., 2001), it was of interest to delineate its mechanism of action.

The BeWo cell line has been widely used as an in vitro model for studying trophoblast intercellular fusion and differentiation, because of its close semblance to the normal physiological process. Under normal culture conditions, BeWo human choriocarcinoma cells grow with cytotrophoblast-like features. When treated with appropriate agents, these cells express typical characteristics of the normal differentiating trophoblast (Speeg et al., 1976; Chou, 1982; Friedman et al., 1984; Taylor et al., 1991). Furthermore, we have demonstrated that induction of differentiation in BeWo cells with either 10 μM Forskolin or 10 ng/ml TGF β1, results in a terminal loss in telomerase activity, in a manner very similar to



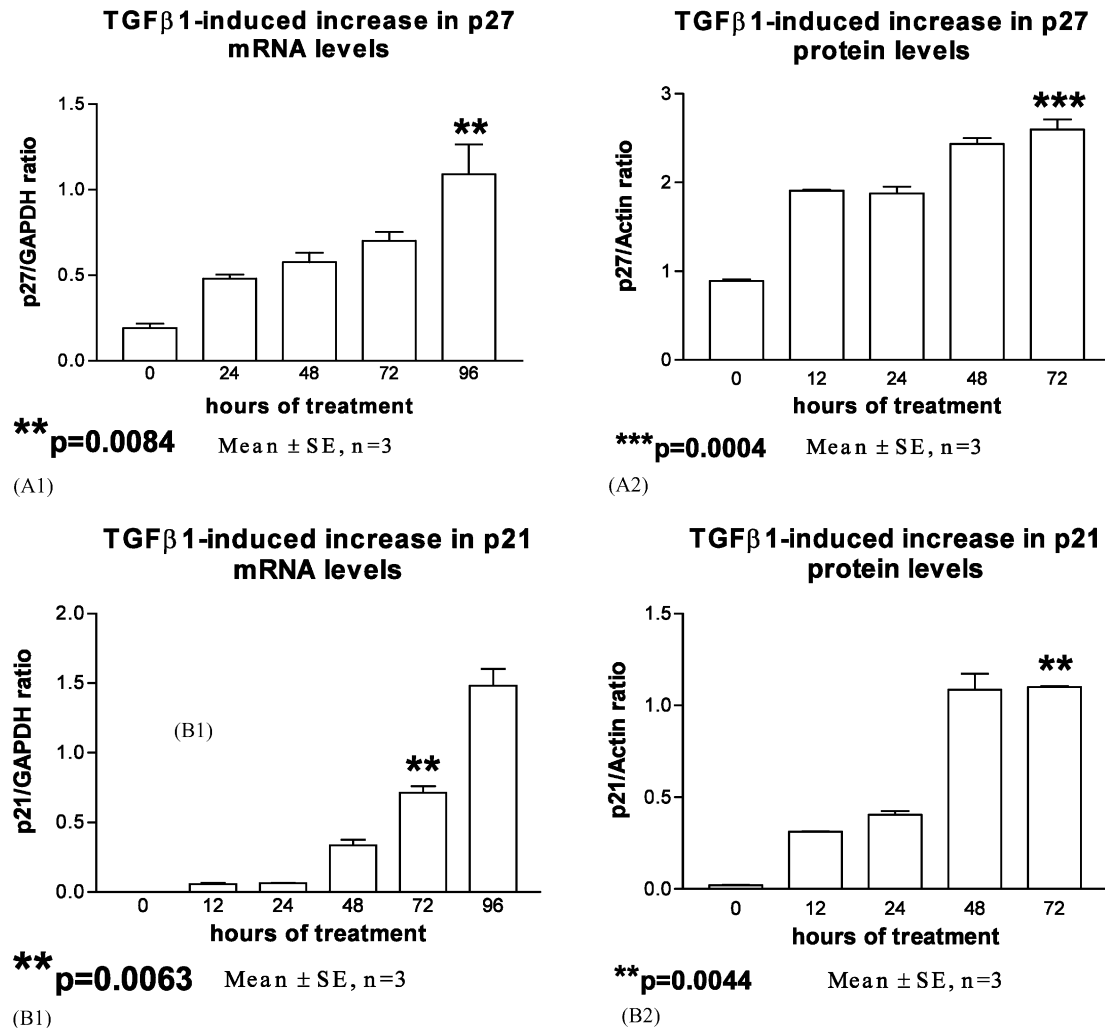
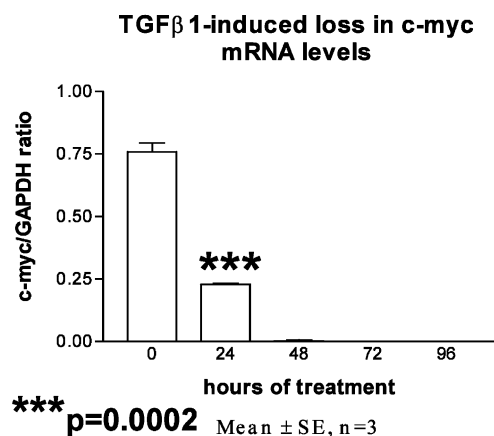


Fig. 5. Regulation of p27<sup>Kip1</sup> and p21<sup>Cip1</sup> expression by TGF β1 in differentiating BeWo cells. (A1) RT-PCR for p27. RNA isolated from 0-h sample, and from differentiating BeWo cells at 24, 48, 72 and 96 h following TGF β1-treatment were subjected to semi-quantitative RT-PCR for p27, with GAPDH amplification as an internal control. Values represent Mean ± S.E. from three independent experiments. (A2) Western Blot analysis for p27. BeWo cells at 0-h, and after 12, 24, 48 and 72 h following TGF β1-treatment, were harvested and analyzed for the expression of p27 protein. The blot was stripped and re-probed for actin, which served as an internal control. Values represent Mean ± S.E. from three independent experiments. Similarly, BeWo cells at 0-h, and at regular intervals following TGF β1-treatment, were harvested and processed for RT-PCR (Panel B1) and Western blot analysis (Panel B2) for p21 expression. Values represent Mean ± S.E. from three independent experiments.

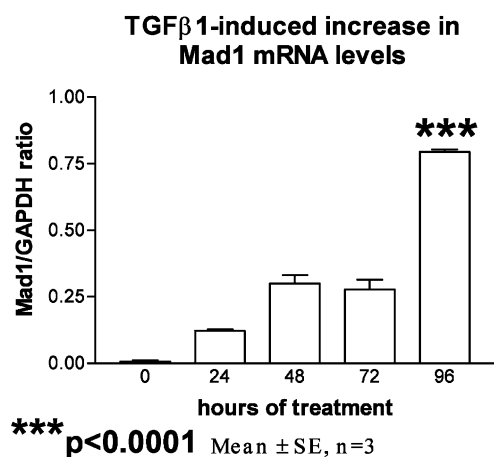
that seen in normal human cytotrophoblasts (Rama et al., 2001). These observations therefore, validate the use of BeWo cells for study of human trophoblast differentiation.

Our results suggest that human trophoblast differentiation is accompanied by a significant loss in c-myc expression, and that TGF β1 may be an important factor governing this event (Fig. 1). In support of this hypothesis is the study by Chen et al. (2001), who showed that TGF β-stimulation of epithelial cells, rapidly induces the formation of a Smad complex that specifically recognizes a TGF β-inhibitory element in the c-myc promoter. Our observations also corroborate earlier reports, which suggest that c-myc expression in the human placenta closely parallels the replicative potential of cytotrophoblasts (Pfeifer-Ohlsson et al.,

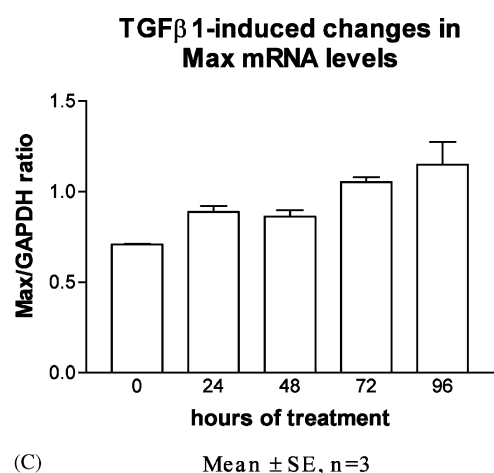
1984; Maruo and Mochizuki, 1987; Rydnert et al., 1987). Interestingly, ectopic expression of the c-myc proto-oncogene in normal human cells was shown to be capable of inducing telomerase activity and extending the life span of these cells in vitro (Wang et al., 1998). Several groups have shown that c-Myc activates telomerase, an effect attributed to direct interaction of c-Myc with the hTERT promoter (Horikawa et al., 1999; Takakura et al., 1999; Wu et al., 1999). These observations led us to compare the expression profile of c-myc and hTERT in our system of differentiating trophoblastic cells. Following 24 h of TGF β1-treatment, there is a total loss in c-Myc expression in differentiating trophoblastic cells (Fig. 1). We have shown earlier (Rama et al., 2001) that TGF β1-treatment in these cells, induces a terminal loss in hTERT expression (that



(A)



(B)



(C)

Fig. 6. TGF  $\beta$ 1-induced effects on the Myc/Mad/Max module during differentiation in BeWo cells. RNA isolated from 0-h sample, and from differentiating BeWo cells at 24, 48, 72 and 96 h following TGF  $\beta$ 1-treatment were subjected to semi-quantitative RT-PCR for c-myc (A; described in detail in the legend to Fig. 1), Mad 1 (B) and Max (C), with GAPDH amplification as an internal control. Values represent Mean  $\pm$  S.E. from three independent experiments.

also precisely parallels a loss in telomerase activity) following 24 h. This suggests that down-regulation of c-Myc may be an important mechanism by which TGF  $\beta$ 1 controls telomerase activity, and therefore indirectly, serves to check trophoblast proliferation.

Our studies also reveal that human trophoblastic differentiation is associated with a substantial loss in the expression of cyclins A2 and E, and that TGF  $\beta$ 1 may be an important factor dictating these molecular controls. Over-expression of cyclin E in human cancers has been correlated with high levels of telomerase activity (Terasawa et al., 1999). It is also suggested that up-regulation of cyclin E may override TGF  $\beta$ -mediated growth inhibitory signals (Kelly and Rizzino, 1999). Interestingly, malignant trophoblastic cells were found to express significantly high quantities of cyclin E when compared to the normal human trophoblast (Kim et al., 2000). It is known that TGF  $\beta$  blocks induction of cyclin A during the G1 phase, and that this event is crucial for inhibition of cell cycle progression (Geng and Weinberg, 1993). These studies, coupled with our observations, suggest that unregulated expression of cyclins A2 and E, may play a significant role in the uncontrolled proliferation and neoplastic transformation of trophoblasts.

The present study also implicates key roles for the Cdk inhibitors p15, p16, p21 and p27, as functional effectors of the TGF  $\beta$ 1-mediated anti-proliferative response in these cells. A myriad of studies underscore the importance of p16<sup>Ink4a</sup> in cell cycle control and tumor suppression (Hall and Peters, 1996; Liggett and Sidransky, 1998). Our study verifies earlier reports, which suggest that p16 gene mutation may be an important factor in the carcinogenesis of trophoblastic cells (Fu et al., 1998). The p15 gene is also reported to be homozygously deleted in a variety of tumor cell lines and primary tumors (Fuxe et al., 2000). The up-regulation of p15 and p16 during human trophoblastic differentiation, therefore, suggests a specific role for these Cdk inhibitors in placental development.

Introduction of autocrine TGF  $\beta$  (Yang et al., 2001) or p21 (Terasaki et al., 1999) into tumor cell lines has been shown to result in the repression of hTERT expression and telomerase activity. In this context, it is pertinent to recall the work of Lin et al. (2000), who have shown that ERV-3 (env-region of the human endogenous retrovirus)-induced differentiation in BeWo cells is also accompanied by an increase in expression of p21. p21 has also been suggested to be involved in mediating the anti-proliferative actions of TGF  $\beta$  in human extra-villous trophoblast cells (Chakraborty et al., 2002). Interestingly, c-Myc has been shown to repress the transcription of p21 (Claassen and Hann, 2000). This perhaps explains the rapid down-regulation of c-Myc that we observe in differentiating human trophoblastic cells. The up-regulation of Cdk

inhibitors p21 and p27, has been observed in several cell types in relation to terminal differentiation, including the human placenta (Kranenburg et al., 1995; Durand et al., 1997; Onishi and Hruska, 1997; Zabludoff et al., 1998; Bamberger et al., 1999). Interestingly, there is an inverse correlation reported between p27 expression and telomerase activity in human malignant glioma cells (Kondo et al., 1998). Their study suggested that p27 protects differentiating telomerase-negative cells against apoptosis. Their study also indicated that loss in telomerase activity could actually be the cause of cellular differentiation. Drawing parallels between this system, and our system of differentiating trophoblastic cells, it is possible that induction of p27 during this process is a protective mechanism that allows for the formation and functioning of the mature syncytiotrophoblast.

Another interesting observation that stems from the present study, is the differing time intervals of mRNA and protein regulation of various factors, following TGF  $\beta$ 1 treatment. For instance, c-Myc protein loss seems to be substantially more than RNA (Fig. 1) during TGF  $\beta$ 1-induced trophoblastic differentiation. On the other hand, increase in p21 protein product appears to be substantially more than the corresponding RNA (Fig. 5B1, B2). Likewise, the increase in p27 mRNA and protein profiles is also not comparable (Fig. 5A1, A2). Although TGF  $\beta$ 1 is known to induce a potent cell cycle arrest by modulating the expression/activity of key cell cycle regulatory proteins, it is important to note that TGF $\beta$  signaling to regulate cell cycle, is also intimately involved with regulation of proteasomal degradation (Zhang et al., 2002a,b). Levels of several of these key factors are controlled by the ubiquitin-proteasome pathway (Zhang et al., 2002a). Therefore, it is possible that these protein levels may be regulated by proteasomal activity as well as by transcriptional regulation. Moreover, proteasomal activity may regulate transcription factors regulated by TGF $\beta$  (Lo and Massague, 1999; Zhang et al., 2002a,b). These two regulatory arms may give rise to the observed difference between protein and mRNA profiles.

Our studies also indicate reciprocal regulation of the transcription factors c-Myc and Mad 1, during human trophoblastic differentiation. Interestingly, recent investigations implicate Mad 1 in the regulation of telomerase activity (Oh et al., 2000; Xu et al., 2001). Mad 1 has been shown to bind the hTERT promoter, at the same sites as c-Myc, and negatively regulate its transcription. Mad 1-mediated repression of the hTERT promoter could be counter-acted by ectopic expression of c-Myc (Oh et al., 2000). Our results suggest that cells with a high replicative potential (and telomerase activity), such as undifferentiated BeWo cells, are characterized by high levels of c-Myc, while Mad 1 expression is generally minimal in these cells. In cellular contexts characterized by minimal expression of hTERT, such as in terminally

differentiated syncytiotrophoblastic cells that are telomerase-negative (Rama et al., 2001), this situation is reversed and c-Myc levels are low while Mad 1 expression is high. This study directly implicates a role for TGF  $\beta$ 1 in the modulation of this network and indirectly, also defines possible mechanisms by which TGF  $\beta$ 1 acts to control telomerase activity in human trophoblasts. In support of this hypothesis is the study by Hurlin et al., (1994), who reported that TGF  $\beta$ -induced differentiation in ML-1 human myeloid leukemia cell line, was simultaneously accompanied by an increase in Mad expression.

While terminal differentiation must necessarily involve the repression of genes provoking proliferation, it must also simultaneously involve activation of genes encoding differentiation-specific proteins. Further, since this program marks an irreversible exit from the cell cycle, there must also be mechanisms to effectively circumscribe the long-term proliferative capacity of these cells. Our studies (Rama et al., 2001 and the present report) define a multi-functional role for TGF  $\beta$ 1 in human trophoblastic differentiation. While on one hand, it induces the expression of differentiation-specific  $\beta$ -hCG, on the other, it induces multiple independent signals that regulate the expression of key components required for progression through the cell cycle, and also check the long-term replicative capacity of these cells by down-regulating telomerase activity. TGF  $\beta$ 1 could thus be an important autocrine/paracrine factor that serves to keep the proliferative potential of tumor-like cells in tight check, and thereby prevent the onset of malignancy. In support of this hypothesis is the study by Perlino et al., (1998), who showed that TGF  $\beta$ 1 expression was found to be drastically reduced in the epithelial component of endometrial carcinomas in comparison to their normal, non-malignant tissue counterparts. Furthermore, loss of TGF  $\beta$  in the skin and skin tumors has been found to increase the risk of malignant conversion (Glick et al., 1993; Cui et al., 1994). Conversely, up-regulation of TGF  $\beta$  could play an important role in pre-eclampsia, which is characterized by insufficient trophoblast invasion. Recent studies using large numbers of patients showed that plasma and placental concentrations of the active form of TGF  $\beta$ 1 were increased in pre-eclamptic pregnancies as compared to those in normal pregnancies (Djurovic et al., 1997; Benian et al., 2002; Bielecki et al., 2002; Chakraborty et al., 2002).

Most importantly, this work highlights the usefulness of the BeWo cell culture model system in understanding the transcriptional reprogramming that accompanies trophoblast cell differentiation. Unlike the other choriocarcinoma cell lines, JEG and JAR, which are refractory to growth inhibition by TGF  $\beta$  (Graham et al., 1994; Xu et al., 2002), our results (Rama et al., 2001 and the present report) seem to suggest that induced

differentiation in the BeWo cell line can recapitulate alterations in gene expression that are characteristic of normal human trophoblast differentiation. The present report extends and further confirms previous studies (Speeg et al., 1976; Chou, 1982; Friedman et al., 1984; Taylor et al., 1991) that also advocate the use of BeWo cells as a valuable model system to analyze mechanisms governing human trophoblast differentiation.

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