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Expression and Transcriptional Regulation of Individual Pregnancy-specific Glycoprotein Genes in Differentiating Trophoblast Cells

S. Camolotto, A. Racca, V. Rena, R. Nores, L.C. Patrito, S. Genti-Raimondi, G.M. Panzetta-Dutari*

Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, CIBICI-CONICET, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, X5000HUA Córdoba, Argentina

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ABSTRACT

Human pregnancy-specific glycoproteins (PSGs), encoded by eleven highly conserved genes, are the major placental polypeptides. Low PSG levels in maternal circulation have been associated with complicated pregnancies. However, expression of each *PSG* gene and their regulation during cytotrophoblast cell differentiation remain poorly explored. Herein, we analyze the expression of five *PSG* genes and demonstrate that they are almost undetectable in undifferentiated trophoblast, but are all transcribed in differentiated cells. Among them, *PSG*1, *PSG*3 and *PSG*5 genes achieve high mRNA levels while *PSG*7 and *PSG*9 are poorly expressed. In addition, total PSG proteins and transcripts markedly increase during trophoblast differentiation, preceding morphological syncytialization and βhCG expression. The 5′ regulatory region contributes to the transcriptional control of *PSG* gene induction in trophoblast cells undergoing differentiation. This responsive region in *PSG*3 maps within a 130 bp promoter sequence, which overlaps the transcription start site and requires a functional Retinoic Acid Responsive Element (RARE) and a GA-binding protein (GABP) consensus site for basal and differentiation-dependent promoter activity, respectively.

Present findings provide novel data for understanding the control of *PSG* gene expression and demonstrate that their proteins and transcripts represent early markers of trophoblast differentiation.

1. Introduction

Cellular differentiation involves the interaction of endogenous gene expression programs and external signals given by hormones, growth factors and cell–cell contacts, among many other factors. During placental development, the trophectoderm of the blastocyst originates the villous cytotrophoblast (CTB) and the invasive extravillous CTB. Villous CTBs proliferate and fuse into the syncytiotrophoblast (STB) layer, which constitutes the uninterrupted, multinucleated epithelium-like surface of the placental villous that separates the maternal blood from the villous interior. The extravillous CTBs aggregate and invade the uterine wall up to the inner third of the myometrium [1]. Abnormalities in this developmental process have been correlated with placental pathologies such as early and mid-trimester pregnancy loss, preeclampsia, intrauterine growth retardation and gestational diabetes [2–4].

Pregnancy-specific glycoprotein (*PSG*) genes are expressed in placenta throughout gestation and are the most abundant placental proteins found in the maternal circulation in late pregnancy. The clinical relevance of measuring PSG in maternal serum was already suggested three decades ago. Low PSG levels in maternal circulation were associated with certain pregnancy complications such as intrauterine growth retardation, preeclampsia and spontaneous abortion [5–8]. In humans, the family is composed of 11 highly similar genes clustered within 700-kilobases on chromosome 19q13.2 [9]. They encode very similar secreted proteins containing a leader peptide (L), one immunoglobulin V-like domain (N), immunoglobulin C2-like domains (A and B) and a short variable carboxy-terminal tail (C). Several studies have determined that PSG proteins cover immuno-regulatory functions. They induce monocytes to synthesize anti-inflammatory cytokines and promote alternative macrophage activation which correlates with the shift from Th1- to Th2-mediated immunological responses [10–12]. PSG protein participation in immune-modulation and T-cell function is reinforced by the fact that elevated PSGs are correlated with improved symptoms of rheumatoid arthritis [13] and multiple sclerosis [14]. Previous studies in placenta have shown that although the intensity of PSG protein immunostaining was higher in preterm than in term placentas [15], total *PSG* mRNA was found to be elevated in term compared to preterm placenta. Furthermore,

* Corresponding author. Tel.: +54 351 4344973; fax: +54 351 4333048.
E-mail address: gpan@fcq.unc.edu.ar (G.M. Panzetta-Dutari).

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a time-dependent increase in PSG1 expression was observed in *in vitro* differentiated STB cells suggesting a role for PSG1 in this process [16,17]. However, the molecular clues involved in the expression of each *PSG* gene during normal villous CTB cell differentiation remain poorly explored.

Human *PSG* promoters are highly similar; all of them lack a TATA-box, a pyrimidine-rich initiator element and large GC regions [18,19]. We have previously demonstrated that *PSG*5 promoter is active in placental as well as in non-placental cell lines, it is down-regulated by promoter selective repressor sequences, and its transcription is largely dependent on a core promoter element (CPE) [20,21]. In addition, we have reported the Sp1 (Specificity protein 1), KLF4 (Krüppel-like transcription factor 4) and RXRα (Retinoid X receptor alpha) contribution to *PSG*5 gene transcription [22–24].

Herein, we demonstrate that: a) the expression of *PSG* genes is an early marker of human trophoblast differentiation into the syncytium pathway; b) *PSG*1, *PSG*3, *PSG*5, *PSG*7 and *PSG*9 transcripts increase in CTBs undergoing differentiation; c) the proximal promoter sequence mediates *PSG* transcriptional activation during trophoblast syncytialization. In addition, present results indicate that RARE is required for basal *PSG*3 transcription and the GABP consensus-binding site contributes to the transcriptional activation associated with trophoblast differentiation.

2. Materials and methods

2.1. Cytotrophoblast isolation, cell culture and differentiation

Tissues from normal term placentas (37–41 weeks of pregnancy) were obtained after natural delivery and processed within 30 min, as previously described [25]. They were collected from unidentified patients with the approval of the Human Studies Committee of the Hospital Privado of Córdoba, Argentina.

CTBs were isolated according to the protocol of Kliman [26]. Isolated trophoblasts (at least 97% cytokeratin 7-positive cells) were plated in keratinocyte growth medium (KGM) (Invitrogen) supplemented with 10% foetal calf serum (FCS) and 100 U/ml penicillin/0.1 mg/mL streptomycin. After 4 h, cells were washed and adhered cells were cultivated in complete growth medium as described [25].

JEG-3, COS-7, and HeLa cell lines were grown in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% FCS. After overnight incubation, cells were cultured for a further 4 days in the presence of 1 μM methotrexate (Mtx) to induce *in vitro* differentiation. Medium was changed every 24 h.

2.2. Immunofluorescence assays

Isolated CTB and JEG-3 cells were cultured on cover slips using supplemented medium as described above and immunofluorescence assays were performed as previously reported [25]. The following primary antibodies were used: polyclonal rabbit anti-PSG (A0131, Dako) 1/100, mouse anti-desmosomal protein (0.045 mg/mL, ZK-31, Sigma Chemical Co.), polyclonal rabbit anti-human chorionic gonadotropin (hCG, A0231, Dako) 1/500 and mouse monoclonal anti-cytokeratin 7 (Dako, Clone OV-TL 12/30). Cells were the incubated with the appropriate species-specific secondary antibodies, red Alexa Fluor 594-conjugated goat anti-mouse IgG or green Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) in a 1/720 final dilution. All antibody incubations were carried out in a humidity chamber for 1 h at 37 °C. Observations were made with an optical microscope (Nikon Eclipse TE2000-U, USA).

2.3. Western blotting

Electrophoresis and blotting was performed as previously described [25]. Briefly, membranes were probed with polyclonal rabbit anti-PSG (A0131, Dako) 1/500 or mouse monoclonal anti-α-tubulin (Sigma–Aldrich) 1/1000. Incubations were performed 1 h at room temperature in 1× TBS-T (100 mM Tris-HCl pH 7.8, 750 mM NaCl and 0.1% v/v Tween 20) and 5% (w/v) non-fat milk. After washing, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibodies (Amersham Bioscience) at room temperature for 1 h diluted 1/5000 in 1× TBS-T.

2.4. Semi-quantitative and qRT-PCR

Total RNA was purified using Trizol (Invitrogen). One microgram of total RNA was reverse-transcribed in a total volume of 20 μl with random primers and M-MLV reverse transcriptase (Promega).

Semi-quantitative and qRT-PCR were performed using the PCR primers listed in Table 1. *PSG* forward primers were designed to prime in the first exon (5′ UTR and leader peptide) and reverse primers within the second exon (leader peptide and N domain) of each *PSG* member. For total *PSG* mRNA amplification, the selected PCR primers were complementary to sequences conserved in all but two *PSG* transcript variants, which lack the N domain. For individual *PSG* gene amplification, each primer pair had at least two mismatches with the non-specific *PSG* targets near the 3′ end. Primers were manually designed with the assistance of the Netprimer software (PREMIER Biosoft International) and Primer Express software (ABI); selected sequences were compared against the human genomic and transcript database with the BLAST program [27] at the NCBI Web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers for *βhCG* were as described in [28]. Conventional PCR conditions were optimized to allow detection in the exponential reaction phase and included an annealing temperature of 60 °C, 1.5 mM Mg^{2+} and the primer concentrations indicated in Table 1. Individual and total *PSG* transcripts were quantified by real-time RT-PCR (ABI 7500 Sequence Detection System, Applied Biosystems) with Sequence Detection Software v1.4. Experiments were performed using 1× SYBR Green PCR Master Mix (Applied Biosystems) and the primer concentrations indicated in Table 1. Specificity was verified by melting curve analysis and agarose gel electrophoresis and was confirmed through direct DNA sequencing (Macrogen Inc, Seul, Korea). Fold change in total *PSG* expression was calculated according to the $2^{-\Delta\Delta Ct}$ method as described [25]. No amplification was observed in PCR reactions containing water or RNA samples incubated without reverse transcriptase during cDNA synthesis.

Relative mRNA abundance among measured *PSGs* was calculated using the inverse of PCR efficiency rised to the power of the difference between the target *PSG* gene Ct and the *cyclophilin A* control gene Ct. Efficiency was determined by $[10^{(-1/\text{Slope})}]$ of the relative standard curve for each amplicon. Relative mRNA abundance was expressed as % of total *PSG* mRNA [29].

2.5. Construction of reporter plasmids

The 5′ proximal regulatory and promoter region of *PSG*3, *PSG*5, and *PSG*7 were isolated from genomic DNA by PCR, purified and cloned into pGL3-basic vector to obtain PB3-luc, PB5-luc and PB7-luc constructs, harbouring sequences corresponding to positions − 613/−49, −605/−49, and −638/−49, respectively (Fig. 4). Different 5′ end positions are due to a different number of TG repeats present in each gene. *PSG*5 promoter region (−251/−49) was amplified and cloned into pGL3-basic to obtain the UB5-luc construct. The *PSG*3 promoter region (−251/−49) was subcloned from PB3-luc by restriction deletion to create UB3-luc, and NB3-luc (−178/−49 of PSG3 promoter) was obtained through PCR amplification, restriction digestion and subcloning into pGL3-basic. mRARE PB3-luc and mGABP PB3-luc are derivative mutants of PB3-luc obtained by PCR-directed mutagenesis of the RARE (GTGTCCTGGGCCTGACCCCG to GTGTCCTGGGCCTtctagaC) and GABP (GCCCCGGGAAGAGGC to GCCCgGtaccGAGGC) binding sites, respectively (consensus sequences are underlined and mutated bases are shown in lower case). All constructs were verified by DNA sequencing.

2.6. Transient transfection assays

Isolated trophoblasts were seeded at a density of 3×10^5 cells per well in 24-well plates. After 12 h, the medium was changed and cells were either immediately transfected or cultivated for additional 36 h before supplementation with gene transfer reagents. Transient transfections were performed in a final volume of 100 μl serum-free OPTI-MEM growth medium (Invitrogen) containing 1 μl of Lipofectamine (2000) (Invitrogen), 1425 ng of the indicated *PSG* reporter construct and 75 ng of the *Renilla* pRL-TK vector (Promega) to correct for transfection efficiency. At 4 h post-transfection, medium was replaced with KGM containing 10% FCS and 5 ng/μL EGF plus antibiotics. After additional 24 h, cellular protein lysates were prepared in 100 μl of the passive lysis buffer. As controls, cells were transfected with pGL3-basic or SV40-promoter luciferase vectors.

JEG-3 cells were seeded at a density of 1×10^5 per well in 24-well plates, cultured for 24 h and afterwards transfected with 2 μl of Lipofectamine (2000), 620 ng of the indicated PSG reporter construct and 30 ng of pRL-TK. After 4 h, medium was replaced with DMEM-10% FCS and antibiotics alone or supplemented with 1 μM Mtx. Protein extracts were prepared 24 h post-treatment. Luciferase and *Renilla* activities were measured in 10 μl of protein extracts on a GloMax-Multi Detection System using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was divided by *Renilla* activity in each cell extract (RLU). Relative promoter activity was calculated as the RLU of each promoter construct divided the RLU of samples transfected with pGL3-basic. Activation fold was expressed relative to the promoter activity of each construct in undifferentiated cells. Since different vector-dependent basal luciferase activity was observed in the differentiated *vs* undifferentiated cultures, they were subtracted from the corresponding construct RLUs before calculating activation fold.

2.7. Statistical analysis

Data are expressed as mean ± SEM of at least three experiments performed in triplicates. Significant differences for each construction activity between the

Table 1
Primer sequences, amplicon size and primer concentrations used in PCR assays.

Primer	Target	Sequence 5′ → 3′	Amplicon size (bp)	Primer concentration in semi-quantitative PCR (μM)	Primer concentration in Q-PCR (μM)
tPSG F	total	CCTCTCAGCCCCTCCCTG			
tPSG R	*PSG*	GGCAAATTGTGGACAAGTAGAAGA	165	0.25	0.1
PSG1F	*PSG1*	TGCACACAGCGCATCAAA			
PSGR		CTCCTCTAGTCCCATCATCTCC	375	0.25	0.25
PSGF	*PSG3*	CCTCTCAGCCCCTCCCT			
PSG3R		GTGAAGGTGAAATGTCCAGTTT	413	0.25	–
qPSG3F	*PSG3*	TGGTCCAGACCTCCCCAGAATT			
qPSG3R		CCAGGAAGATGTCCTGTTCC	282	–	0.1
PSGF	*PSG5*	CCTCTCAGCCCCTCCCT			
PSG5R		TACTCCTCTAGTCCTATCACCTCG	393	0.25	–
qPSG5F	*PSG5*	CAAGTCACGATTGAAGCCCT			
PSG5R		TACTCCTCTAGTCCTATCACCTCG	300	–	0.25
PSG7F	*PSG7*	CACACAGCATATAACCTGGAAA			
PSG7R		ATCCTGTGTCTTCCTGGGTG	327	0.25	–
PSG7F	*PSG7*	CACACAGCATATAACCTGGAAA			0.25
qPSG7R		GGCAAATTGTGGACAAGTAGAAGA	158	–	0.1
PSG9F	*PSG9*	CCTCCCAGCCCCTTCC			
PSG9R		GGTGTAGGTTCCTGCATCCTT	355	0.25	–
qPSG9F	*PSG9*	TCCTGCTCACAGCATCACTTTT			
PSG9R		GGTGTAGGTTCCTGCATCCTT	310	–	0.1
GAPDHF	*GAPDH*	GGTGAAGGTCGGAGTCAACG			
GAPDHR		GATCTCGCTCCTGGAAGATGG	236	0.25	–

undifferentiated *vs* differentiated condition or between two constructions were identified using two-sided Student's *t*-test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. PSG transcripts and proteins increase in trophoblast cells undergoing differentiation

Two different trophoblast cell systems were employed, primary CTBs and choriocarcinoma JEG-3 cells. *In vitro* differentiation of term villous CTBs is a useful model to study CTB syncytialization [30]. However, we aimed to employ a cell line-based system, which offers the advantage of unconstrained cell access to perform experiments under controlled conditions. It had been demonstrated that the treatment of JEG-3 with Mtx inhibits cell growth and increases hCG production [31]. Therefore, we test if it was an appropriate model to study differentiation-dependent *PSG* gene expression.

Morphological syncytialization of treated JEG-3 cells was confirmed by desmosomal protein staining, which progressively disappeared at the intercellular boundaries as syncytium-like structures were formed in a time-dependent manner (Fig. 1A). In undifferentiated JEG-3 cells, total *PSG* transcripts and proteins were low or undetectable but Mtx-induced differentiation resulted in a time-dependent increase (Fig. 1B and C). To confirm that activation of *PSG* expression was associated with trophoblast cell differentiation and was not due to a direct effect of Mtx, similar experiments were done in COS-7 and HeLa cells. Even though Mtx treatment promoted cell size increase, there were no signs of either cellular fusion or *PSG* transcript and protein activation (data not shown).

In freshly isolated CTBs, *PSG* transcripts were very low or undetectable and markedly increased after 96 h of *in vitro* differentiation (Fig. 2A, top panel). Simultaneous evaluation of total *PSG* and *βhCG* transcripts revealed that both were expressed after 24 h; instead only *PSG* mRNA was clearly detected after 16 h (Fig. 2A). A 101 ± 29 -fold induction (mean ± SEM) of total *PSG* transcripts was observed on CTB RNA samples purified from four different placentas and differentiated for 96 h. PSG protein expression also augmented upon differentiation (Fig. 2B) reaching expression levels remarkably higher than those observed in differentiated JEG-3. Immunofluorescence assays revealed PSG protein expression in the context of CTB morphological differentiation. Staining was undetectable in 4 h CTB cultures but became evident after 16 h, before formation of the syncytium-like structures, and further increased with culture time (Fig. 2C). In addition, βhCG immunostaining confirmed that PSG protein expression also precedes βhCG synthesis (Fig. 2D). Altogether, these results indicate that total *PSG* transcript and protein induction represents an early biochemical marker associated with trophoblast cell differentiation.

3.2. mRNA expression of individual PSG genes in differentiated trophoblasts

Individual *PSG* member expression study is demanding due to their high degree of sequence identity and the lack of specific antibodies for each PSG protein. Herein, we studied the expression of *PSG*1, *PSG*3, *PSG*5, *PSG*7 and *PSG*9 which are evenly distributed along the entire *PSG* cluster [9]. Specific transcripts for each of the analyzed *PSG* genes were detected in differentiated JEG-3 and CTBs while they were undetectable or had low expression level in undifferentiated cells (Fig. 3).

Relative abundance levels were evaluated in differentiated CTBs through qRT-PCR. *PSG*1, *PSG*3 and *PSG*5 transcripts were more abundant than *PSG*7 and *PSG*9 mRNAs, and although their abundance varied among samples, the distribution into high and low-expressed mRNAs was maintained (Table 2). The high or low-expressed genes did not group together as more similar when

a Clustal alignment of 3000 nucleotides upstream of the first codon was performed (data not shown). Indeed, *PSGs* share more than 93% nucleotide sequence identity and the most dissimilar region corresponds to a stretch of (TG)n repeats. These motives have a propensity to undergo structural transitions and have been shown to modulate transcription in several genes with an inverse correlation between repeat length and transcriptional levels. In most cases, it was observed that repeats of $n \geq 12$ units exert a down regulatory effect on transcription [32]. Nevertheless, present results do not support a potential regulatory function in *PSGs* since *PSG*7 and *PSG*9 contain repeats of more than 12 units and are expressed at lower levels than *PSG*5 with only 6 repeat units but *PSG*1 and *PSG*3, which include 12 and 20 repeat units respectively, were among the highly expressed transcripts (Table 2). Also, transcript level differences among these genes were not associated with a pattern of sequence conservation within the putative regulatory elements previously described for *PSG*5 [22,24]. However, we cannot rule out that subtle nucleotide differences may participate in controlling the overall transcription rate and therefore relative abundance of each *PSG* gene. The data suggest that a fine-tuning complex mechanism that may include specific long-range acting chromatin factors, transcriptional regulation and transcript stability controls the expression of each *PSG* gene member.

3.3. Differentiation and transcriptional activation of PSG genes

In order to establish the contribution of transcriptional control to *PSG* induction during differentiation, we analyzed the promoter activities of approximately 600 bp of the 5′ proximal regulatory regions of two highly expressed (*PSG*3 and *PSG*5) and one low-expressed (*PSG*7) genes. Even though expression of the endogenous transcripts was undetectable in undifferentiated JEG-3 cells, *PSG*

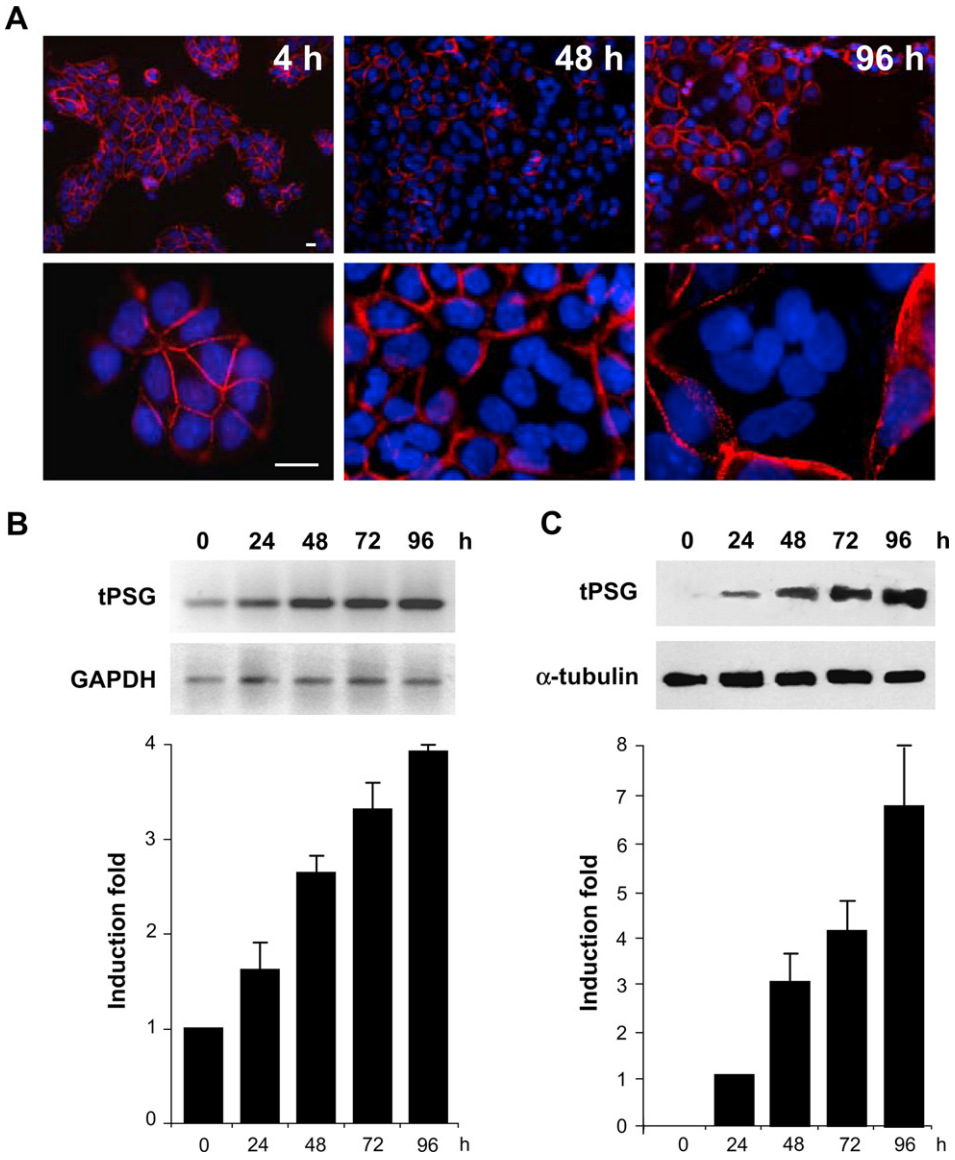

Fig. 1. Total *PSG* transcripts and proteins increase in JEG-3 cells undergoing differentiation. A) Immunostaining of fixed cells with anti-desmosomal protein (red) and nuclear counterstaining with Höechst (blue). Bar = 10 μm. Original magnifications: top panel ×200, bottom panel ×1000. B) Semi-quantitative RT-PCR of total *PSG* transcripts (tPSG). C) Western blot for total PSG proteins (100 μg). The blots shown correspond to one representative experiment of three with similar results. Induction fold was calculated after dividing the density value obtained for *PSG* mRNA or protein expression by the value obtained for the corresponding internal control (*GAPDH* mRNA and α-tubulin, respectively). Basal expression was arbitrarily set as 1 and values represent the mean ± SEM of three independent experiments. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

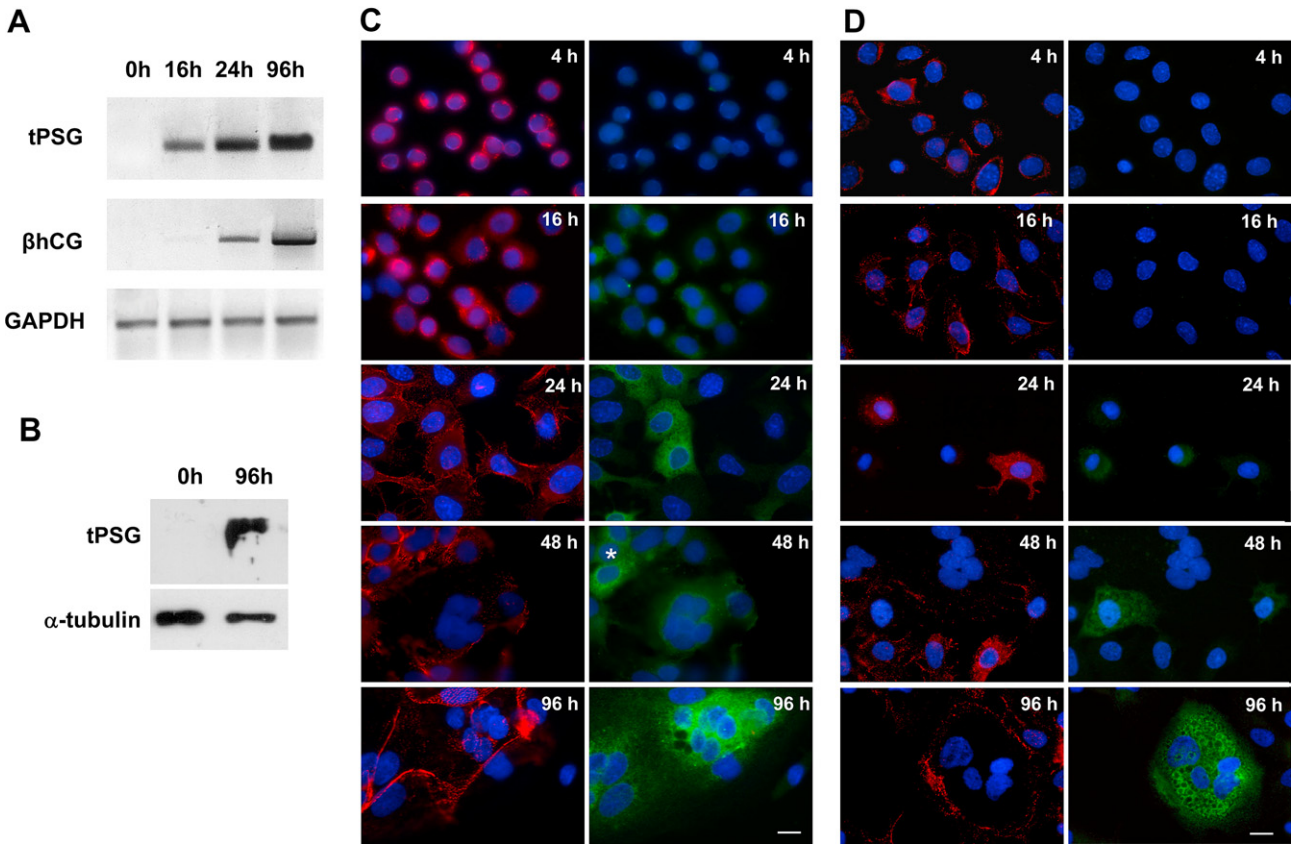

Fig. 2. Total *PSG* transcripts and proteins increase in differentiated CTBs. A) Semi-quantitative RT-PCR of total *PSG* and *βhCG* in CTBs differentiated for the indicated hours. A representative experiment of three independent experiments performed from CTBs isolated from three placentas is shown. B) Western blot assay for total PSG proteins (0 h, 100 μg or 96 h, 10 μg). C and D) Immunofluorescence detection of PSG (C) or βhCG (D) proteins (green), desmosomal protein (red) and nuclei (blue) in CTBs cultured during 4, 16, 24, 48 and 96 h. Asterisk, non-fused CTB expressing PSG proteins. Bar = 10 μm. Original magnification: ×1000. [For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.]

promoter constructs were active in this cell condition (Fig. 4A). When JEG-3 cells were induced to differentiate each *PSG* construct exhibited a small but significant increase in luciferase activity, but promoter activity of the *PSG* unrelated SV40 promoter remained constant (Fig. 4B). Thus, response elements located at the 5′ regulatory region mediate specific transcriptional activation upon differentiation of JEG-3 cells.

Transcriptional control was further investigated in primary CTB cultures. All *PSG* constructs were active when they were transfected in early cultures (12 h) displaying different relative luciferase activity. Pair-wise comparison of UB3-luc *vs* PB3-luc, and UB5-luc *vs* PB5-luc, indicated the presence of a functional repressor element located upstream nt −251 (Fig. 4C), as it had been described for *PSG5* in non-placental cells [20]. In CTBs differentiated for 48 h, PB3-luc and PB5-luc, as well as their UB3-luc and UB5-luc deletion constructs, had higher activities than those displayed in early cultures; in contrast, promoter activity of the low-expressed *PSG7* gene and of the *PSG* unrelated SV40 promoter remained unaffected (Fig. 4D). Further deletion of *PSG3* sequences up to position −178 (NB3-luc) revealed that, at least for *PSG3*, the differentiation responsive element/s should be located within the 130 bp promoter sequence (−178/−49). This region contains several consensus DNA binding sites. Among them are a RARE motive and a putative binding site for the Ets-family transcription factor GABP. To explore the contribution of these elements to the enhanced *PSG* transcription observed in differentiating trophoblasts, we compared the functional activity of the wild-type PB3-luc with the RARE or GABP mutated constructs (Fig. 4E). In early CTB cultures, RARE mutation nearly abolished promoter activity. Instead, there was no significant difference in the transcriptional activity of the GABP mutated construct compared to wild-type PB3-luc (Fig. 4E, grey bars). In more differentiated CTBs, wild-type PB3-luc was clearly activated but the RARE mutant remained inactive and the activity of the GABP mutant did not increase compared to that of PB3-luc in early CTBs (Fig. 4E, black bars). Therefore, the RARE binding site is required for basal promoter activity while the GABP binding site is involved in the induction of *PSG3* transcription during differentiation.

In sum, these results confirm that proximal promoter elements contribute to the transcriptional increase of *PSG* expression

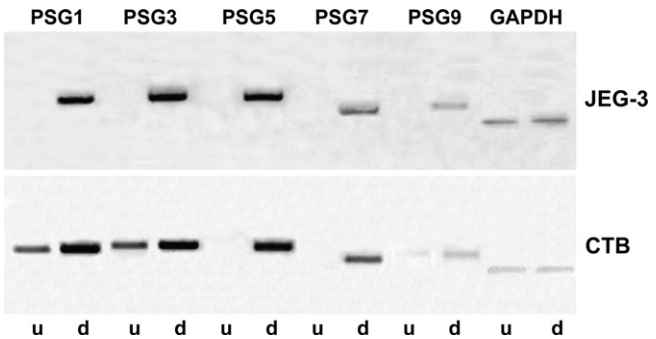

Fig. 3. *PSG1*, *PSG3*, *PSG5*, *PSG7*, and *PSG9* transcripts are up-regulated upon trophoblast differentiation. RT-PCR for the indicated *PSG* and *GAPDH* genes were performed with RNA from JEG-3 cells cultured (d) or not (u) in differentiating conditions for 96 h, and of isolated CTBs cultured for 4 h (u) or 96 h (d).

Table 2
Relative mRNA abundance of *PSG* genes in differentiated cytotrophoblasts.

Gene	% Relative PSG gene abundance[a]		Putative regulatory motives					
	Median	Range	(TG)n	RARE	CPE	FP1	FP3	FP4
PSG1	6.23	5.37–31.85	20	TGTCCTGGGCCTGACCC	CCC**TG**CCC	GGGCGT**T**	CAGGGACC**T**G	CACAATGGGG
PSG3	11.12	6.34–17.32	12	TGTCCTGGGCCTGACCC	CCCC**G**CCC	GGGC**A**TG	CAGGGACC**T**G	CACAATGGGG
PSG5	20.46	15.53–29.18	6	TGTCCTGGGCCTGACCC	CCCCACCC	GGGCGTG	CAGGGACCCG	CACAATGGGG
PSG7	0.63	0.06–2.25	22	TGTCCTGGGCCTGACCC	CCCCACCC	GGGCGTG	CAGGGACC**T**G	CACAATGGGG
PSG9	0.05	0.01–0.31	18	TGTCCTGGG**A**CTGACCC	CCCC**G**CCC	**A**GGC**A**TG	CAGGGACC**T**G	CACAATGGGG

[a] Values obtained in samples from five different placentas. Putative regulatory motives previously identified in PSG5 are as follows: (TG)n, stretch of TG repeats; RARE, retinoic acid responsive element; CPE, core promoter element; FP1–4, footprint elements 1, 3 and 4. Nucleotide differences with respect to PSG5 are highlighted in bold.

associated to trophoblast cell differentiation into the syncytium pathway.

4. Discussion

The present study was undertaken to gain insight into the expression and transcriptional regulation of *PSG* genes in differentiating trophoblast cells. Our results confirm and further extend previous reports regarding *PSG* expression [15,16,18,33]. Herein, we demonstrate that total PSG proteins and transcripts markedly increase in isolated CTBs undergoing differentiation into the syncytium pathway. This induction precedes morphological syncytialization and *βhCG* expression. Present results support the proposal of *PSG* as an early biochemical marker of STB formation. Moreover, considering that placental chorionic villous turnover entails the shedding of syncytium material into the maternal circulation and since this process is altered in pathologic

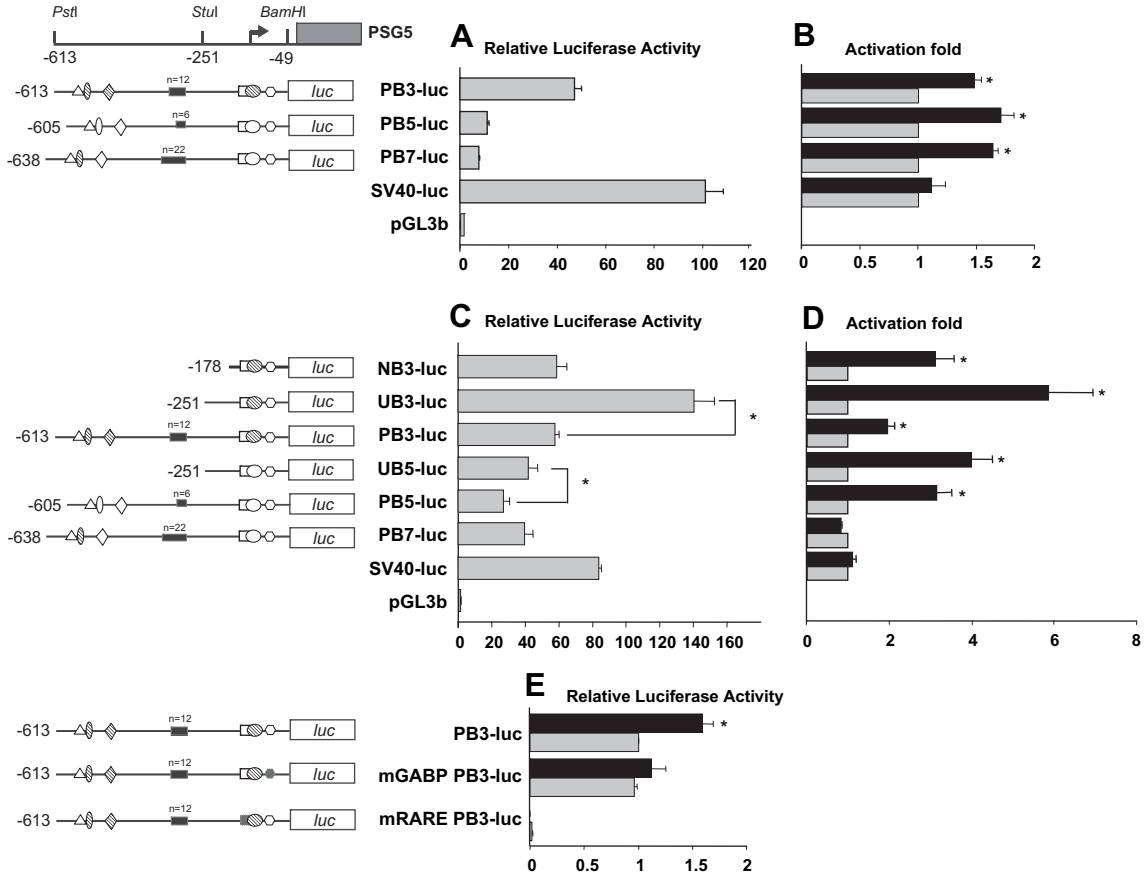

Fig. 4. Transcriptional activities of *PSG* constructs in JEG-3 and CTB cells. Reporter constructs with putative functional regulatory elements are shown on the left, CPE (○), RARE (□), FP1 (◇), FP3 (◯), FP4 (△), (TG)n repeat (■) and GABP (○). Hatched symbols indicate subtle sequence differences with those previously identified in *PSG5* [22,24]. Mutated elements are in grey. A and B) JEG-3 cells transfected and treated (black bars) or not (grey bars) with 1 μM Mtx. C, D and E) early (12 h, grey bars) or late (48 h, black bars) CTB cultures transfected with the indicated constructs. Results are expressed as relative luciferase activity respect to pGL3-basic (A and C) or respect to the PB3-luc activity in early CTBs (E). For easier comparison between the reporter activities of each construct in both differentiation conditions, results are expressed as activation fold respect to the undifferentiated cultures (B and D). Data from three to five experiments performed in triplicate are shown (mean ± SEM). * Statistically significant difference ($p < 0.05$) between the indicated constructs or between each construct in both cells conditions.

pregnancies [34], total *PSG* transcripts could be regarded as a candidate plasma biomarker of placental function.

As suggested, different *PSG* members may have different functions [35]. Thus, it is useful to know which members are expressed in trophoblast cells in order to shed light on our understanding of *PSG* function in human pregnancy. It has been largely assumed that all *PSG* genes are expressed in placenta. Nevertheless, gene expression analyses have provided controversial results. For instance, expression profiling studies in normal and preeclamptic placenta tissues have identified *PSG*4, *PSG*5 and *PSG*7 as the highly expressed genes in term pregnancies [36,37]. A microarray survey of 6900 genes in human trophoblast cells differentiating into the syncytium pathway identified *PSG*1 as the most up-regulated gene [17]. However, this study did not address the relative abundance levels among individual *PSG* genes. Herein, we designed gene specific primers and established amplification conditions to measure *PSG*1, *PSG*3, *PSG*5, *PSG*7 and *PSG*9 transcript levels. Each of the selected genes belongs to one of the blocks that characterize the family cluster [9]. Present data suggest that the whole *PSG* locus is activated in CTBs that differentiate into the syncytium pathway, although they reach different abundance levels. It is interesting to note that syncytium-like trophoblast cells express very little *PSG*9 mRNA and, conversely, up-regulation of *PSG*9 expression, but not any other *PSG* member, was found in colorectal carcinogenesis [38]. Thus, *PSG*9 may have a diverse function related to carcinogenesis instead of placental immunomodulation.

The 5′ proximal regulatory regions of the highly expressed *PSG*3 and *PSG*5 genes exhibited enhanced transcriptional activity in both differentiation systems, while *PSG*7 gene was activated in JEG-3 but not in CTB differentiated cells. In addition, *PSG* transcriptional induction was higher in CTB than JEG-3 cells. Consequently, JEG-3 cells appear as a partially convenient model to perform initial studies on *PSG* expression. Accordingly, the putative differentiation responsive region was further delimitated through experiments performed on primary cultures. In this cell system, a 130 bp promoter sequence overlapping the transcription start site was sufficient to mediate differentiation-associated *PSG*3 activation. This sequence includes a GABP consensus-binding site which was required for promoter activation in differentiated CTB and a RARE motif necessary for promoter activity in differentiated and undifferentiated CTB. Notably, both elements are highly conserved in all *PSG* genes. Our data suggest that members of the RXR and *Ets* transcription factor families potentially contribute to basal and differentiation-dependent *PSG* transcription, respectively. In this context, *Ets* transcription factors as Ets-2 (*Ets* transcription factor subfamily member 2) and Pea3 (polyomavirus enhancer activator 3) are expressed in villous trophoblast, participate in normal placentogenesis and regulate trophoblast gene expression [39,40]. RXRα is required for normal trophoblast development and it is a known regulator of STB hormone synthesis [41–43]. Indeed, we had previously demonstrated that RARE mediates *PSG*5 transcriptional activation by RXRα in JEG-3 and most significantly, the responsiveness to 9-cis retinoic acid was enhanced in JEG-3 cells upon differentiation [24]. Therefore, it is possible to hypothesize that the RARE motif is involved not only in basal promoter activity but also in *PSG* activation upon trophoblast differentiation; further studies should address this possibility. In addition, it would be interesting to consider the role of Sp1, since the CPE element mediates *PSG*5 transcriptional activation by Sp1 [22] and the Sp1/Sp3 ubiquitous transcription factors are induced during trophoblast fusion and are critical for STB specific gene expression [44,45].

Even though our results demonstrate the contribution of the 5′ proximal control region to *PSG* gene activation, it does not explain by itself their remarkable increase in expression. Hence, transcriptional activation by other regulatory sequences, epigenetic control and/or transcript stability should be considered to fully understand the molecular mechanisms that mediate enhanced expression of *PSG* genes during trophoblast cell differentiation.

In sum, this report provides novel data regarding the expression of human *PSG* genes as early markers of trophoblast differentiation and demonstrates the contribution of transcriptional mechanisms to *PSG* gene activation associated with trophoblast syncytialization.

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