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TRANSFORMING GROWTH FACTOR BETA HAS DUAL EFFECTS ON MMP9 AND uPA EXPRESSION IN HTR-8/SVneo HUMAN TROPHOBLASTIC CELL LINE

El factor de crecimiento transformante beta tiene efecto dual en la expresión de MMP9 y uPA en la línea celular de trofoblasto HTR-8/SVneo

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ABSTRACT

Invasion of trophoblast into endometrium is vital for successful pregnancy development. MMP9 and uPA are key proteases in this process, but it is still not clear the regulation of its expression by Transforming Growth Factor Beta (TGF-β), a known negative regulator of trophoblast invasion. We evaluated the effect of TGF-β on the transcriptional expression of uPA and MMP9 over time, in HTR-8/SVneo trophoblast cells cultured with or without 0.5 % fetal bovine serum, via RT qPCR. The involved transcription factors and signaling pathways were analyzed in silico, using Proscan, Enrich, PCViz and WikiPathway. Results showed that TGF-β temporarily regulates the expression of uPA and MMP9. Serum modified the nature of TGF-β's effects on uPA expression, from negative without serum to positive with it, showing opposite effects on MMP9 expression. *In silico* analysis evidenced different transcription factors for each protease, some belonging to TGF-β signaling pathway, and crosstalk with MAPK and Wnt/β-catenin pathways. The TGF-β dual role is discussed proposing that serum affects the cellular context. Transcriptional regulation of MMP9 and uPA by TGF-β is differential and depends on serum presence and evaluation time.

Keywords: Computational biology, matrix metalloproteinase, placenta, plasminogen, polymerase chain reaction.

RESUMEN

La invasión del trofoblasto al endometrio es vital para el correcto desarrollo del embarazo. Las proteasas MMP9 y uPA son claves en este proceso, pero aún no es clara la regulación de su expresión por parte del Factor de Crecimiento Transformante beta (TGF-β), conocido por sus acciones no invasivas sobre el trofoblasto. En este trabajo evaluamos el efecto del TGF-β sobre la expresión transcripcional de uPA y MMP9 en células de la línea de trofoblasto HTR-8/SVneo cultivadas con o sin suero fetal bovino al 0,5 %, mediante RT qPCR. Se analizaron in sillico los potenciales factores de transcripción y vías de señalización involucradas empleando Proscan, Enrich, PCViz y WikiPathway. Los resultados muestran que el TGF-β regula temporalmente la expresión de uPA y MMP9. El suero modificó la naturaleza del efecto del TGF-β sobre la expresión de uPA, de negativo en ausencia de suero a positivo en presencia de este, presentando efectos opuestos para la expresión de MMP9. El análisis in sillico evidenció diferentes factores de transcripción para cada proteasa, algunos pertenecientes a la vía de señalización del TGF-β, y un entrecruzamiento con la vía MAPK y Wnt/β-catenina. Los resultados sugieren que la regulación transcripcional de MMP9 y uPA por parte del TGF-β es diferencial y depende de la presencia de suero y tiempo de evaluación.

Palabras clave: Biología computacional, metaloproteinasa de matrix, placenta, plasminógeno, reacción en cadena polimerasa.

INTRODUCTION

Extravillous cytotrophoblast (EVT) is an integral tissue of the placenta which invades the uterine endometrium and the maternal spiral arteries, remodeling them (Lunghi *et al.*, 2007; He *et al.*, 2017). An adequate EVT invasion is vital for blastocyst implantation and successful pregnancy development. When this process is modified, some pathologies occur such as preeclampsia, intrauterine growth restriction, and gestational trophoblastic diseases. This invasive process requires the expression and activation of extracellular proteases, highlighting matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA). An increase in the expression of MMPs and uPA is a uniform phenomenon in diverse cancer types, and it is correlated with tumor progression and metastasis (Friedl and Wolf, 2003; Turunen *et al.*, 2017; Mahmood *et al.*, 2018). Amongst MMPs, MMP2 and nine have been the most characterized enzymes in cancer cases, and MMP9 plays a relevant role in invasive trophoblast processes (Staun-Ram *et al.*, 2004; Cohen *et al.*, 2006; Zhang *et al.*, 2018). On the other side, it has been demonstrated that the activator system of plasminogen uPA plays an important role in the regulation of invasion and migration of trophoblast (Lala and Chakraborty, 2003; Zheng *et al.*, 2018). Even though EVT cells behave as metastatic (Ferretti *et al.*, 2007), their invasion *in vivo* is transitory, spatially confined and highly regulated by a plethora of factors (Cohen *et al.*, 2006; Menkhorst *et al.*, 2016; Li and Shao, 2017), including the transforming growth factor beta (TGF-β), which has negative effects on trophoblast proliferation, migration and invasion (Chakraborty *et al.*, 2002; Lunghi *et al.*, 2007; Yi *et al.*, 2018) and promote its differentiation (Cheng *et al.*, 2018).

TGF-β causes a significant reduction in secretion and activity of uPA, and induces the expression and secretion of its inhibitor PAI-1 and the tissue inhibitor of metalloproteases TIMP-1 and -2, which inhibit the extracellular matrix degradation performed by the MMPs (Lala *et al.*, 1998). It has been seen that TGF-β reduces the expression and secretion of MMP9 in primary cultures of trophoblast isolated from first-trimester placenta (Meisser *et al.*, 1999), in explants of first-trimester placenta (Lash *et al.*, 2005), in the choriocarcinoma cell line JEG-3 (Karmakar and Das, 2002) and in the trophoblast cell line NPC (Zhao *et al.*, 2006). Nevertheless, other studies in primary cultures of the first-trimester cytotrophoblast have demonstrated that TGF-β stimulates expression and secretion of MMP9 (Shimonovitz *et al.*, 1996). The immortalized first-trimester EVT cell line HTR-8/SVneo was established and described as a model that partially responds to the regulatory actions of TGF-β, with a decrease in secretion and activity of uPA without affecting the invasion level (Graham *et al.*, 1993), being proposed as a model for the study of human placental function and tumor progression (Lala *et al.*, 2002). However,

more recent works show that TGF-β promotes invasion of HTR-8/SVneo cells in advanced culture passages, involving expression changes in members of its signaling pathway (Yanzhen *et al.*, 2014). Furthermore, TGF-β1 upregulated cadherin-11 expression in HTR-8/SVneo through activating SMAD2 and SMAD3 signaling pathways inducing epithelial to mesenchymal transition (Cheng *et al.*, 2018). This apparent contradiction in the effect of TGF-β on protease expression and cellular invasion of the trophoblast requires additional studies that consider the expression over time and *in vitro* culture conditions, specifically the treatment in presence or absence of fetal bovine serum (FBS).

In previous studies with HTR-8/SVneo cells cultured for 24 hours with or without fetal bovine serum (0 %, 5 %, and 10 %), we have seen a change in protein profiles which suggests a metabolic adaptation due to serum absence (Novoa-Herran *et al.*, 2016).

Additionally, it has been seen that the effects of IGF-I and insulin on the invasion of cellular trophoblast models are serum-dependant (Mandl *et al.*, 2002).

Considering the above, it is relevant to study the effects of TGF-β on the expression of MMP9 and uPA in different culture conditions and over time, since expression of these proteases is an inflection point in the regulation of EVT invasion. In this work, transcriptional expression of uPA and MM9, as a response to different doses of TGF-β, was evaluated by means of quantitative PCR in HRT-8/SVneo cell line, in presence or absence of 0.5 % FBS and over time; possible transcription factors and involved signaling pathways as well, were identified by a bioinformatic analysis.

MATERIALS AND METHODS

Cell line and culture conditions

The immortalized cell line of human extravillous trophoblast HTR-8/SVneo was donated by Dr. Ángela Cadavid (Universidad de Antioquia, Colombia). This cell line was developed from a culture of first-trimester human placenta explant and was immortalized by transfection with a cDNA that codes for the simian virus 40 (SV40) large T-antigen (Graham *et al.*, 1993). These cells exhibit a high proliferation index and share several phenotypic similarities with parental HTR-8 cells, including invasive abilities *in vitro*. Cells were manipulated from passage 15 to 20 (own records) and cultured at 37 °C in humidified atmosphere with 5 % CO_2 in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10 % FBS (Gibco, Invitrogen), 2 mM L-glutamine (Gibco, Invitrogen), and 40 µg/mL gentamicin (Genfar). Cells were seeded in 60 mm culture dishes and cultured in supplemented medium for one to two days until 60-80 % confluency was achieved, were starved overnight in serum-free medium, after that stimulus was performed in the mentioned doses and times.

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Treatment

Cells were stimulated with transforming growth factor β isoform 1 (hBA-112, Santa Cruz Biotechnology Inc., Receptor grade) in a concentration of 10 ng/mL and for 0, 6, 12, 18 and 24 hours. Additionally, TGF-β1 in concentrations of 0.1, 1, 5, 10 and 20 ng/mL was used. Different concentrations up to 20 ng/mL as indicated for each experiment. These treatments were performed in serum-free RPMI medium or concomitant with 0. 5% FBS using serum-free RPMI medium or with 0.5 % FBS as control, respectively.

RNA extraction

Each culture dish was treated with 1 mL of TRIzol reagent (Invitrogen, USA) according to the instructions of the manufacturer. The cells were lysed with the reagent, generating a monophasic solution composed of phenol-guanidium isothiocyanate, and extracting total RNA in the aqueous phase by phase-partitioning with chloroform. RNA was precipitated with isopropanol, washed with 75 % ethanol in RNase-free water and solubilized in 30 µL of RNase-free water. The concentration and purity of the obtained RNA were determined by spectrophotometry (Pharmacia Biotech Ultraspec, 2000) and its integrity was verified by agarose gel electrophoresis.

cDNA synthesis by retro-transcription

The reaction was carried out in a Perkin Elmer PC100 thermocycler (Perkin Elmer, USA). Each reaction was prepared in a volume of 20 µL, mixing 2 µg of RNA with 0.50 mM dNTPs and 50 ng of random primers in a first step. This mixture was heated to 65 °C for 5 min, cooled and then 40 units of RNase inhibitor, 4 µL of 5X transcription buffer, 5 mM dithiothreitol (DTT) and 200 U of SuperScript III RT (Invitrogen) were added. Afterward, the mixture was homogenized and centrifuged, proceeding with the following program: 25 °C for 10 minutes, 50 °C for 50 minutes, 70 °C for 15 minutes and preservation at 4 °C.

Evaluation of expression by real-time PCR

The cDNA was diluted 1:10 with RNase-free water. Additionally, a pool composed by the mixture of 1 µL of cDNA of each sample was prepared, at different serial dilutions (1:5, 1:25, 1:125, 1:625, 1:3125) and a calibration curve for the genes *18S* (Hrs 18S), *β-actin*, *PLAU* (uPA) and *MMP9* was built. Real-time PCR reactions were performed in a BioRad CFX96 thermocycler (BioRad Laboratories Inc), using a reaction mixture: 1 µL of cDNA (1:10 dilution or calibration curve dilution as corresponds), 1.5 µL of Master Mix iQ™ SYBR® Green (BioRad Laboratories Inc), 0.35 µL of RNase-free water and 0.15 µL of 10 µM gene-specific primers for each gene. The primers were designed based on the reported entries in GenBank and the sequences in direct (D) and reverse (R) sense were: *MMP9*: D: AACCAATCTCACCGACAGG, R: CGACTCTCCACGCATCTC; *PLAU* (uPA): D: AGCAGAGACACTAACGACTTCAG, R: CTTA

CTCACACTTACACTCACAGC; *Hrs-18S*: D: ATGTGGTGTTGA GGAAAGC, R: TACTGGCGTGGATTCTGC; *β-actin*: D: GCG TGA CATTAAGGAGAAG, R: GAAGGAAGGCTGGAAGAG. Forty cycles were performed with 10 s at 95 °C, 20 s at 56 °C and 20 s at 72 °C. The fluorescence threshold (Ct) was calculated with the software CFX Manager version 1.6 (BioRad Laboratories Inc) and a melting curve was made once the last amplification cycle finished as a criterion to detect unspecificity and dimer formation. Each determination was performed by triplicate, obtaining the relative expression normalized to genes *18S* and *β-actin* used as reference genes, accordingly to the data treatment and error propagation suggested by Hellemans *et al.*, (2007). Finally, the normalized relative expression (NRQ) and the obtained standard deviation (SD) was re-scaled, normalizing the data to each biological control and following the propagation of error formulas.

Statistical analysis

For simple comparisons between treatments and controls, unpaired Two-tailed T-test was used. For comparison between treatments and times or stimuli doses a two-way analysis of variance (ANOVA) was performed, and Bonferroni test for subsequent analysis (biological replicate, n=3, $p<0.05$).

Bioinformatic analysis of the promoter region and regulation by transcription factors

The 5' sequences flanking genomic sequences of *MMP9* and *PLAU* (uPA) were analyzed using the PROSCAN v 1.7 software (Dan Prestridge, University of Minnesota; http://www-bimas.cit.nih.gov/molbio/proscan/) applying default parameters. For the sequence of the *MMP9* gene, 14654 base pairs were processed and for the sequence of *PLAU*, 13398 base pairs were processed. The predicted results of the promoter region were complemented with a transcriptional analysis employing Enrich analysis tools (Chen *et al.*, 2013). The transcription factors that bind to the promoter of the genes were obtained from the experimental databases ChIP ChEA 2015 (ChIP-x Enrichment Analysis) and ENCODE TF ChIP-seq 2015 (Encyclopedia of DNA Elements). Transcriptions factors with binding sites in the promoter were inferred against the databases TRANSFAC, JASPAR PWMs, and Genome Browser PWMs. A consensus between the different analysis of the transcription factors that bind to the promoter of MMP9 and/or PLAU was performed.

To determine which of these factors are part of the TGF-β signaling pathway, we used Cytoscape 3.3.0 (Shannon *et al.*, 2003) to link the list of transcription factors in the *TGF beta Signaling Pathway* network of Wikipathways App (Kutmon *et al.*, 2014).

Protein interaction networks were created using PCVIz of Pathway Commons (Cerami *et al.*, 2011), for TGF-β with MMP9 and uPA, as a group and separately, including interactions that control state change and expression, in

order to detect intermediaries that could mediate functional relationships between the cytokine and proteases.

RESULTS

The presence of serum differentially affects the expression of MMP9 and uPA as a response to different doses of TGF-β
The effect of TGF-β on the expression of uPA and MMP9 proteases was evaluated in cells of the immortalized trophoblast-derived cell line HTR-8/SVneo, by RT-PCR in real time, normalizing the results against the 18S ribosomal unit and the β-actin genes, before the specific normalizations of each analysis.

In general, the nature and magnitude of the effect of TGF-β on gene expression change with the dose, the presence of serum and with the evaluation time (Fig. 1). No significant differences were observed among the different TGF-β doses, although this factor affects the expression of each protease, being significant for the expression of MMP9 with serum (Fig. 1B; 0.1 ng/mL at 12 and 24 h, 1 and 5 ng/mL at 12 h) and the expression of uPA without serum

(Fig. 1 C; 10 and 20 ng/mL at 24 h) and with serum (Fig. 1 D; 1 and 20 ng/mL at 12 h). Specifically, the treatment of TFG-β with 0.5 % FBS caused a decrease between two and four times the expression of MMP9 at 12 hours, depending on the doses (0 vs 0.1 ng/mL 3.57-fold p=0.0192; vs 1 ng/ml 4.17-fold p=0.0151; vs 5 ng/mL 3.23-fold p=0.0221; vs 10 ng/mL 2.63-fold n.s.), while at 24 hours a decrease higher than five times with the dose 0.1 ng/mL (0 vs 0.1 ng/mL 5.56-fold p=0.0490) was recorded. In the case of uPA we found a two to three times higher increase as a response to TGF-β with FBS at 12 hours (0 vs 1 ng/mL 3.15-fold p=0.0306; vs 20 ng/mL 2.74-fold p=0.0323). When the treatment was performed in the absence of serum, the expression of uPA decreased as a response to TGF-β, being significant at 24 hours (0 vs. 10 ng/mL 2.78-fold p=0.0005; vs. 20 ng/mL 1.79-fold p=0.0062).

When evaluating the influence of serum on the effect of the treatment, the expression of these genes as a response to this cytokine differed significantly between with or without FBS (Fig. 2), specially for uPA which presented an expression profile in response to TGF-β that was clearly distinctive
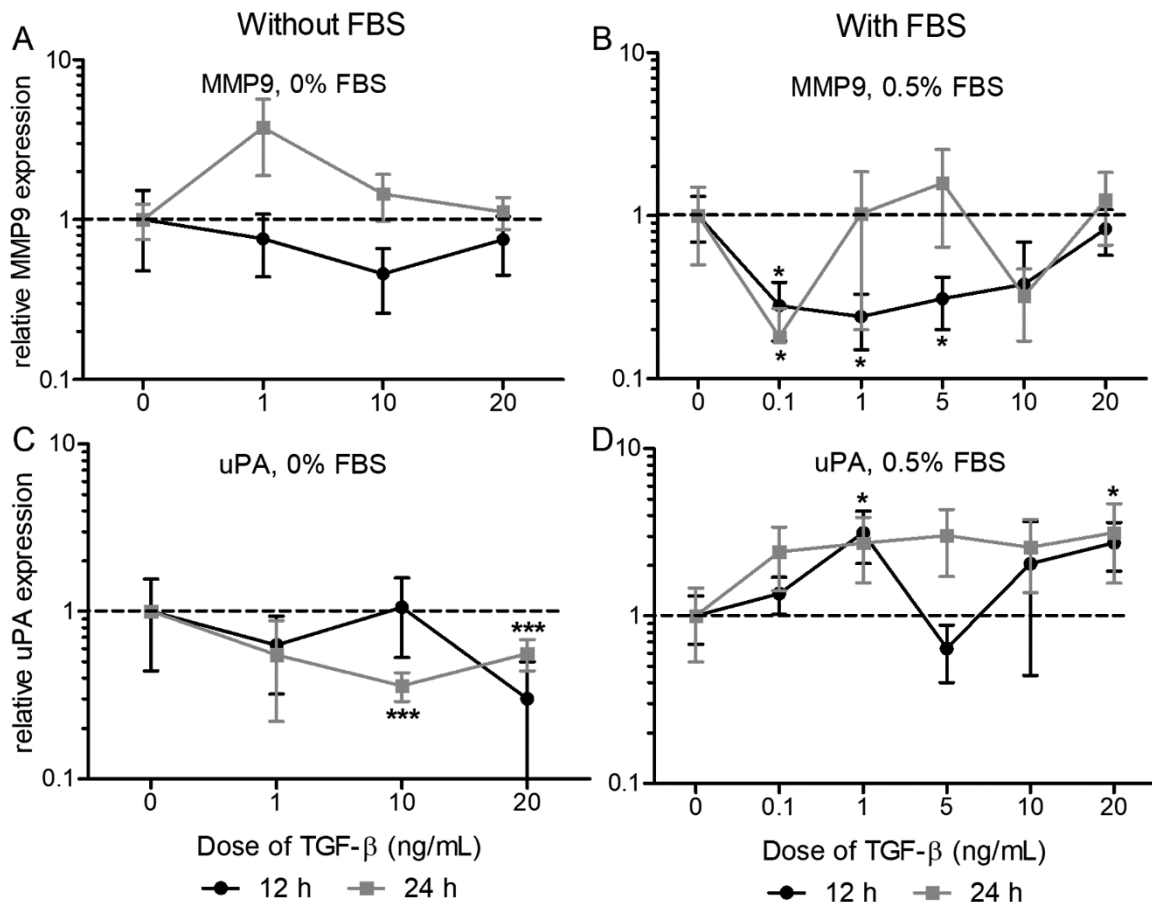

Figure 1. Effect of TGF-β on the expression of MMP9 and uPA. We evaluated the response to TGF-β doses until 20 ng/mL at 12 and 24 hours, without serum (A and C) or with 0.5% FBS (B and D) on the expression of MMP9 and uPA respectively. Relative expression to 18S and β-actin genes, normalized against control (TGF-β 0 ng/mL). Unpaired Two-tailed T-test compared to control (n=3 p>0.05, ** p<0.001). Relative expression axis in logarithmic scale.

between with or without serum treatment and independent on the dose or the evaluated time. Noticeably we observed that presence or absence of serum modified in a significant manner the nature of the effect of TGF-β, changing from decreasing the expression of uPA in absence of serum to an increase in presence of 0.5 % FBS both at 12 hours (Fig. 2 C, 1 ng/mL t=3761 $p<0.01$; 20 ng/mL t=3641 $p<0.01$) and at 24 hours (Fig. 2 D, 1 ng/mL t=3.196 $p<0.05$; 10 ng/mL t=3.255 $p<0.05$; 20 ng/mL t=3.783 $p<0.01$). In the case of MMP9, at 12 hours the TGF-β negatively affected the expression, consistently and homogeneously, independent on the presence of serum; but at 24 hours TGF-β promoted the expression in the absence of serum while diminished it with serum (Fig. 1 A and B, Fig. 2 B 1 ng/mL t=4.152 $p<0.01$), observing a concomitance between treatment and serum as in the case of uPA, but with opposite effects.

Expression kinetics: The effect of TGF-β on the expression of MMP9 and uPA as a function of time

To evaluate the expression dynamics of the genes *MMP9* and *PLAU* (uPA) over time, both in a basal form and as a response to TGF-β, HTR-8/SVneo cells were cultured in RPMI medium with 0.5 % FBS, with or without 10 ng/mL TGF-β and mRNA was extracted every 6 hours leading to analysis times of 0, 6, 12, 18 and 24 hours. The employed dose was 10 ng/mL, widely used in the literature. Cells cultured for the same time in medium with 0.5 % FBS were used as a control. Initially, to carry out the statistical analysis, data were compared and normalized against the respective control for each time. Finally, the relative expression was normalized against zero time to obtain the kinetics showed in Figure 3.

The effect of TGF-β on the expression of MMP9 and uPA over time varied in accordance with the studied gene. When comparing with the corresponding control at each time, TGF-β, in general, decreased the expression of MMP9 being statistically significant at 12 hours (2.63-fold t=3.136 $p<0.05$) and 24 hours (3.13-fold t=6.228 $p<0.001$). Unlike uPA, for which TGF-β reduced expression at 6 hours but caused an increase at 12, 18 and 24 hours, being statistically significant in the latter (2.58-fold t=4.676 $p<0.001$) (data not shown).
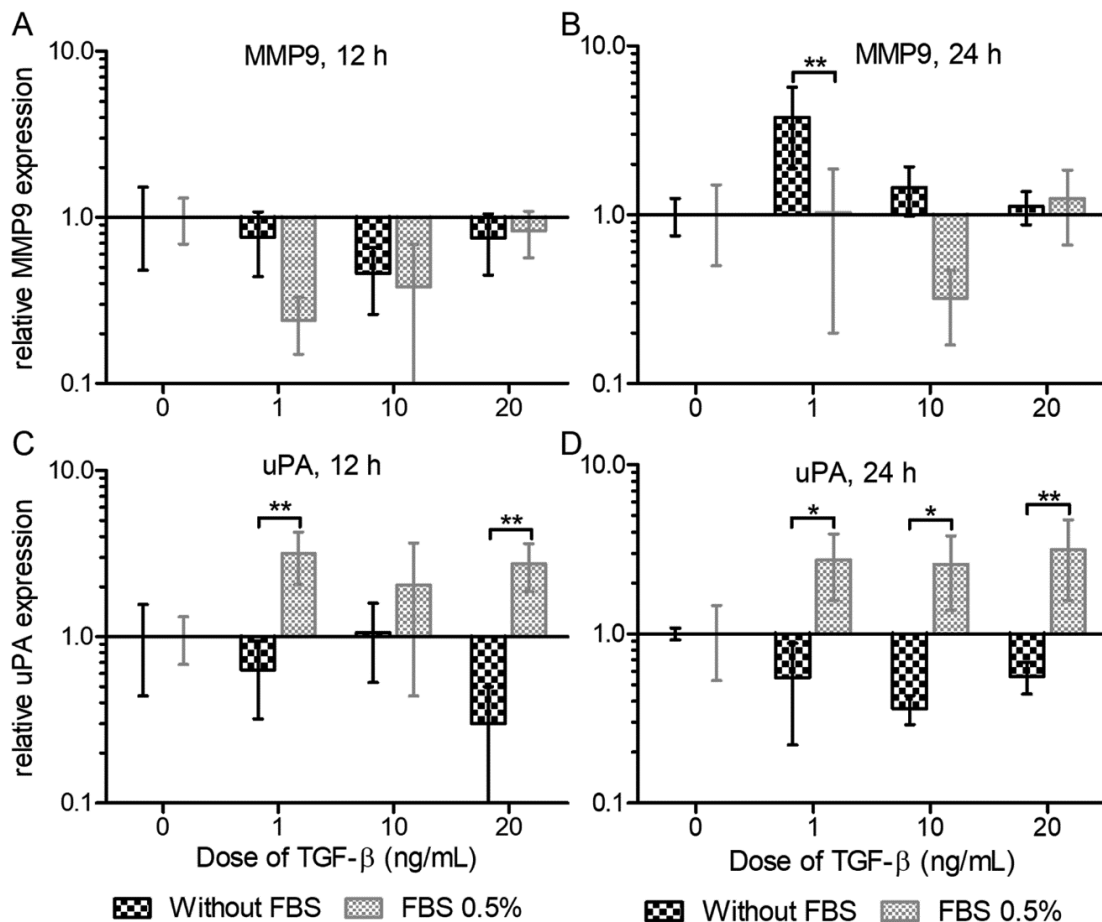

Figure 2. Effect of TGF-β relative to the presence of serum on the expression of MMP9 at 12 (A) and 24 hours (B), and of uPA at 12 (C) and 24 (hours). We evaluated TGF-β doses of 1, 10 and 20 ng/mL at 12 and 24 hours, concomitant treatment with or without 0.5% FBS. Relative expression to 18S and β-actin genes, normalized against control (TGF-β 0 ng/mL). Two-way ANOVA test to compare between with and without conditions (n=3, *$p<0.05$, ** $p<0.01$, *** $p<0.001$). The relative expression axis is presented in Log 10 scale.

Additionally, when normalizing the relative expression over time against expression at zero time, a dual effect on the proteases expression over time was detected, following a sinusoidal pattern, with out-of-phase peaks –coordinated but opposed between the genes. In this way, while at 6 hours TGF-β increased the expression of MMP9 (2.5-fold) and decreased the expression of uPA (1.74-fold), at 18 hours TGF-β increased the expression of uPA (6.61-fold) and decreased the expression of MMP9 (18.34-fold) (Fig. 3).

Analysis of promoters and regulation of transcription factors

The observed differences on the TGF-β transcriptional regulation of the genes under study, could be the result of a differential signaling pathway activation, and therefore, on the corresponding transcription factors. A bioinformatics analysis of the promoters and transcription factors of *MMP9* and *PLAU* (uPA) genes was performed using the Proscan and Enrich tools. MMP9 and PLAU genes are in different chromosomes (ch20q13.12 and chr10q22.2, respectively) and despite a common group of transcription factors was found, most of them were specific for each gene. Figure 4A contains a final representation, indicating the transcription factors that bind, or probably bind, to the promoter of each gene.

Linking these transcription factors with the TGF-β pathway reported by Wikipathway (Fig. 5), some common elements as Fos/Jun were observed. Fos/Jun form a multimeric complex with Smad2-3/Smad4, as well as Myc, EP300, Sp1, and JunD, which coordinate the expression of both proteases. Among the reported transcription factors for PLAU but not for MMP9, are included CREBBP, Ets1, and E2F4, highlighting ATF2, atf3 and MEF2A, which are activated by phosphorylation by MAPK14. As for MMP9, SMAD4, TP53,

and JunB are reported as regulators of this gene but not of PLAU (Fig.4A), suggesting they only follow the TGF-β/SMADs canonic pathway (Fig. 5).

Using the Pathway Commons an analysis was performed to evaluate the interaction network between TGF-β and MMP9 or uPA, as well as the network between the cytokine and both proteases (Fig. 4B). The role of Jun as a common transcription factor for both proteases was confirmed. We observed in the network from TGF-β to MMP9, that SMAD7 – that mediates the signaling from the ligand connects to CTNNB1 or β-catenin, a key downstream component of the canonic Wnt pathway. In the case of uPA network, a remarkable interaction was observed between TGF-β and members of the MAPK pathway as MAPK1 and MMAPK3, reinforcing the results obtained, when crossing the transcription factors that regulate uPA in an exclusive form, with the TGF-β pathway from WikiPathways.

DISCUSSION

In the present work, we found that the presence of fetal bovine serum (0.5 % FBS) affects in a significant way the nature of the effects of TGF-β on the expression of uPA in cells of the HTR-8/SVneo trophoblast-derived cell line. Treatment with TGF-β in conjunction with 0.5 % FBS increased the expression of uPA at 12 and 24 hours, while in the absence of serum, the expression was decreased. In the case of MMP9, we also observed a serum-dependent response at 24 hours, but in an inversely way: in the absence of serum TGF-β increased the expression of MMP9, whereas in the presence of serum a variable response was observed. On the other hand, at 12 hours a decrease was observed in MMP-9 expression, without an apparent effect of serum. This finding suggests that the transcriptional regulation of
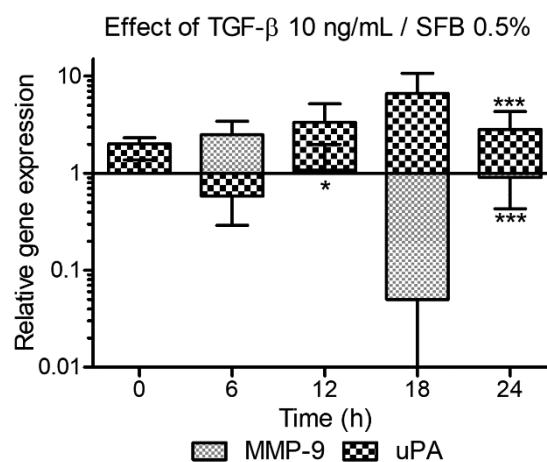

Figure 3. Kinetics of the effect of TGF-β over the expression of MMP9 and uPA. We evaluated the expression of MMP9 and uPA at 0, 6, 12, 18 and 24 hours after treatment with 10 ng/mL TGF-β with 0.5% FBS. Statistical analysis: Two-way ANOVA, comparing the NRQ of the treatment (T = TGF-β) against control (C = TGF-β 0 ng/mL, 0.5% FBS) at each time for each gene: (T_{xh}/C_{xh}) (n=3, $*p<0.05$, $** p>0.01$, $*** p<0.001$). The bars correspond to the expression relative to the 18S and β-actin genes, normalized against its control (TGF-β 0 ng/mL) at each time and then at time zero: (T_{xh}/C_{xh}) / (T_{0h}/C_{0h}).
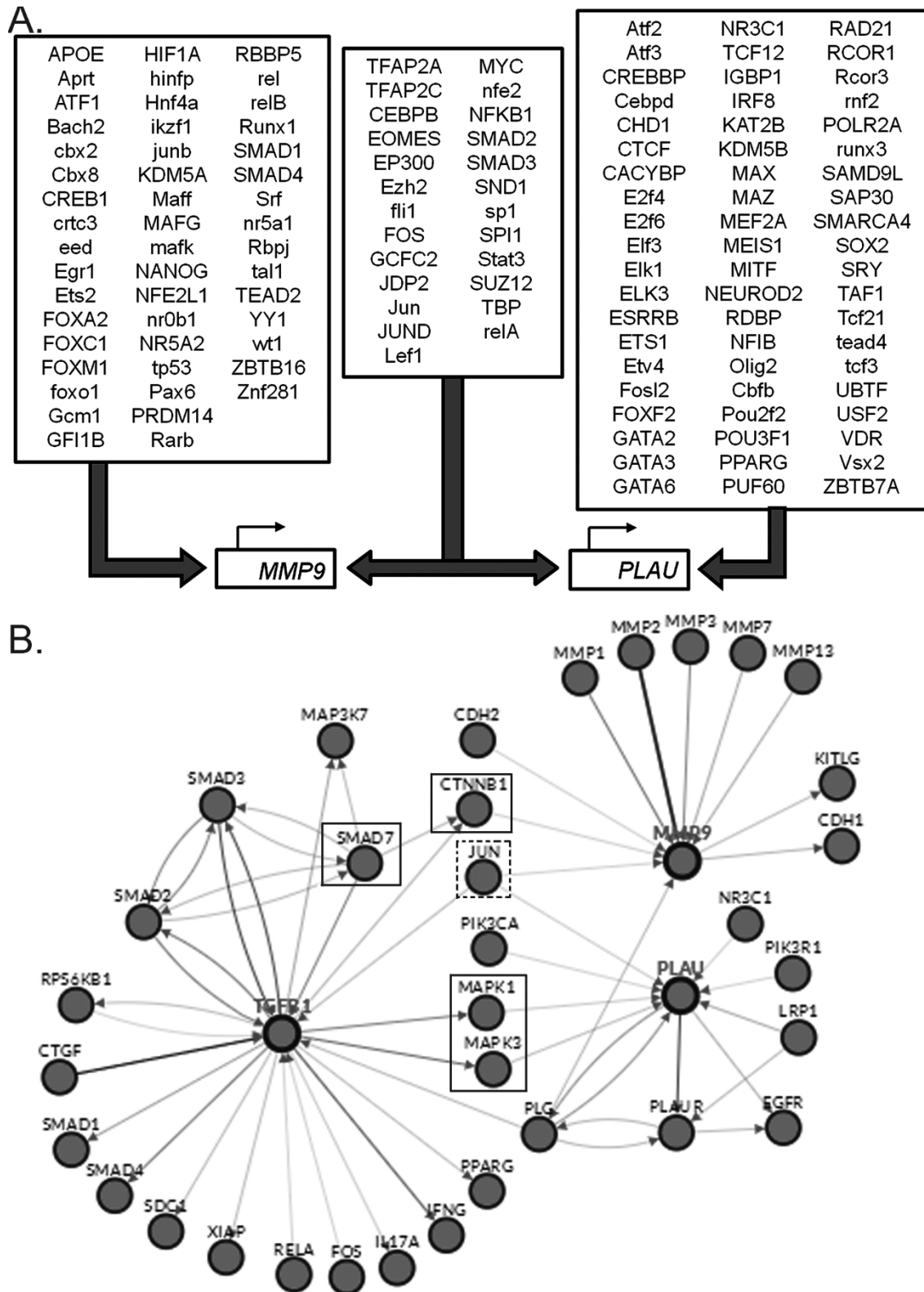
Figure 4. A. Transcription factors with binding sites in of *MMP9* and *PLAU* (uPA) genes promoters. Consensus list created from Proscan v. 1.7 and Enrich analysis (ChIP ChEA 2015, ENCODE TF ChIP-seq 2015, TRANSFAC and JASPAR PWMs and Genome Browser PMWs). **B. Analysis of Pathways commons pathways.** Obtained network between the TGF-*β*, MMP9 and PLAU genes, obtained with PCViz and addition of 34 nodes. Blue connections: state changes, green connections: expression. Dotted line frames: connector nodes shared between TGF-*β* and both proteases. Continuous line frames: connector nodes exclusive to MMP9 or PLAU.
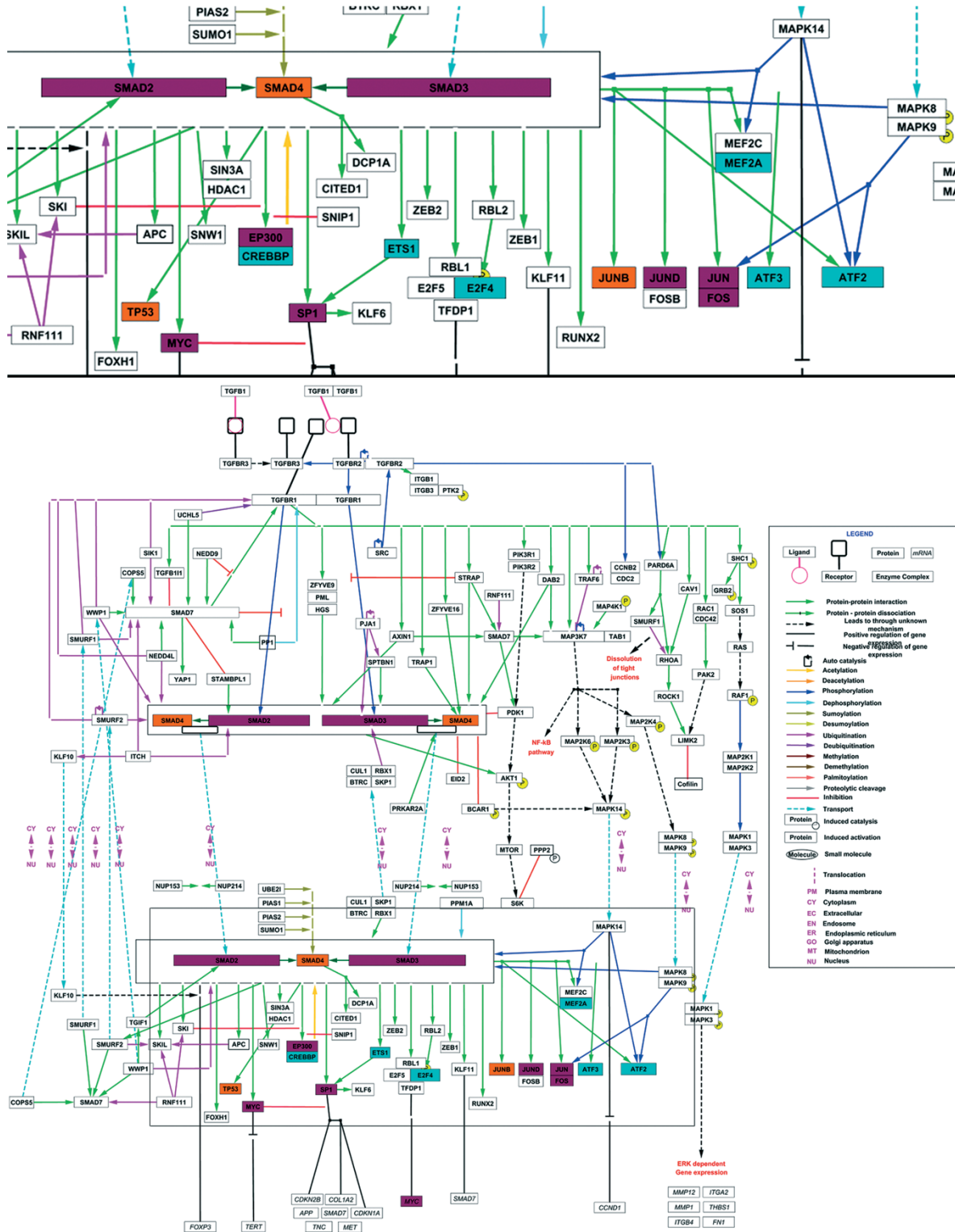
Figure 5. TGF-β signalling pathway and transcription factors involved in the expression of MMP9 and PLAU (uPA). Inferred transcription factors that regulates the expression of MMP9 and PLAU (purple nodes), only MMP9 (orange nodes) or only PLAU (blue nodes), belonging to the TGF-β signalling pathway reported by WikiPathways.

uPA can be more dependent on the presence of serum, and therefore on the environmental conditions, in comparison with MMP9 expression.

In addition to the influence of serum, our results also showed that the effects of TGF-β **on** the expression of MMP9 and uPA changed with time, evidencing a dual effect

of TGF-β with sinusoidal kinetics. A coordinated regulatory pattern for both genes was obtained, but with out-of-phase peaks, due to the opposed effects of TGF-β: while at 6 hours post-stimuli, TGF-β inhibits the expression of uPA and increases the expression of MMP9, at 12 hours the effect is the opposite. This phenomenon could be explained in part, by the activation of different signaling pathways and their corresponding transcriptional mediators operating at their kinetics. Another factor may arise from a possible modification of the culture conditions, due to nutrient uptake and conditioning of the medium with autocrine factors that altogether modify the characteristics of the culture medium. These changes could influence the response to TGF-β, generating other input signals, which explain the changes in response to TGF-β due to the presence of serum and over time, highlighting the influence of microenvironment or cellular context.

Studies on the effects of insulin and IGF-I, acting separately or in synergy with the serum, on the secretion of chorionic gonadotropin beta (hCG), proliferation and invasion of BeWo, JAR and JEG-3 choriocarcinoma cell lines, a strong synergistic effect with serum was revealed (Mandl et al., 2002). Other studies have demonstrated that a high glucose concentration inhibits the activity of uPA and in vitro invasion of HTR-8/SVneo cells, with no observed effects on the levels of MMP2, MMP9, PAI-1 or uPAR (Belkacemi et al., 2005). The observed dependence of the signaling of TGF-β on the culture conditions and its versatility, can be explained based on its dual behavior, as tumor suppressor in certain cell types and as pro-metastatic in many invasive tumors (Pardali and Moustakas, 2007), and whose effects greatly depend on the cellular context (Ikushima and Miyazono, 2010).

The trophoblastic tissue has been considered as "pseudo-malignant" or "physiologically metastatic" because its cellular abilities (Ferretti et al., 2007; Li and Shao, 2017) and similar to solid tumors, the placenta has several trophoblast subtypes, which vary with the gestational stage (Liu et al., 2018); even more, the HTR-8/SVneo cell line after immortalization by transfection with SV40 virus large T-antigen (Graham et al., 1993) can present additional features, being proposed as a tumor progression model (Lala et al., 1998). In spheroid formation assay, HTR-8/SVneo spheroids showed trophoblast progenitor cell-like characteristics and are suggested that these spheroids contain the progenitor cell characteristic of repopulating activity in a near in vivo model (Weber et al., 2013). Nevertheless, TGF-β has been studied only as a tumor suppressor in these models, based on its classic anti-proliferative, anti-invasive and anti-migratory actions (Chakraborty et al., 2002; Lala et al., 2002), without considering its dual effect according to the biological context in which it performs its actions. Taking into account the HTR-8/SVneo cell line characteristics and its origin, as well as the dual role or dichotomy of TGF-β, it is not surprising to observe the opposed effects of this factor

on the expression of MMP9 and uPA, key proteases in the invasive process of both trophoblast (Lala and Chakraborty, 2003; Staun-Ram et al., 2004; Cohen et al., 2006; Zheng et al., 2018) and metastatic tumors (Friedl and Wolf, 2003; Mahmood et al., 2018). Even though the presence of 0.5 % FBS can modify the cellular status and therefore the response capacity to exogenous factors, based on the performed study, it is not possible to discern the role that FBS plays.

Until now, no systematic studies about the expression of these two genes in response to TGF-β over time, or about the influence of serum during the treatment had been performed. In this work, we found a differential response to TGF-β as a function of time, for both uPA and MMP9 genes, exhibiting dual kinetics, which partially agrees with the literature. In general, it has been seen that TGF-β decreases the secretion and activity of uPA in the HTR-8/SVneo cell line (48 h, serum-free ExCell medium) (Graham et al., 1993) and in first-trimester placenta explants (24h, serum-free ExCell 300 medium) (Lash et al., 2005), simultaneously decreasing the secretion and activity of MMP9 in primary first-trimester cytotrophobast cultures (4 days, serum-free DMEM medium) (Meisser et al., 1999), and in placenta explants (24 h, serum-free ExCell 300 medium) (Lash et al., 2005), and confirming a reduction in the expression and activity of uPA and MMP9 in the choriocarcinoma cell line JEG-3 (10 ng/mL, 24 h) (Karmakar and Das, 2002).

Nevertheless, in primary first-trimester cytotrophobast cultures we observed an increase in the expression of MMP9 at 8 hours and its activity at 12 hours, as a response to TGF-β1 (10 and 20 ng/mL, serum-free RPMI 1640 medium) (Shimonovitz et al., 1996); similarly, an increase in MMP9 and 2 as a response to TGF-β was observed in early cytotrophobast cultures and the choriocarcinoma cell line BeWo (Yudate et al., 1996). This agrees with an increase in the expression of MMP9 at 6 hours in 0.5 % FBS medium or at 24 hours in serum-free medium, highlighting the duality of TGF-β and its context-dependent actions.

The disparity in the previous results can be due not only to the specific culture conditions –such as cellular model, medium type, the presence of serum, dose and stimulus time– but also to the type of technique used for transcript measuring. In the present study, the genic expression was evaluated in a time range from 6 to 24 hours by qPCR, which despite being implemented as a relative comparison, has a much higher sensibility and allows to detect small changes in transcript abundance, making possible to determine that TGF-β has a different effect at short times (6 hours). At longer culture times, the medium is conditioned by trophoblast-secreted factors that could have an autocrine effect, increasing the complexity in both the signaling and the results interpretation.

The difference in the TGF-β transcriptional regulation on each gene may be explained in part by the different sets of transcription factors found for each gene, especially those

belonging to the TGF-β signaling pathway, as Smad4, TP53 and JUNB for MMP9, and CREBBP, Ets1, E2F4, ATF2, ATF3 and MEF2A for PLAU. This difference can also be due to a differential signaling activation, as the bioinformatic analysis suggests. It is necessary to explore possible crossed signaling events, including co-activation of the MAPK pathway. Previously it was seen that TGF-β promotes invasion of HTR-8/SVneo cells in advanced culture passages, involving a reduction in the expression of TβRI, Smad3, and Smad4, increase of Smad7 (Yanzhen *et al.*, 2014), and induction of expression of MMP2 through Smad2, with no Smad 4 involvement (Lin *et al.*, 2006), and more recently TGF-β was reported to induce invasion in JEG-3 cells and up-regulates the MMP-9 expression (Huang *et al.*, 2017). Cellular context-specific factors whose action depends on environmental conditions can generate signaling dynamics and spatial-temporal regulations (Kholodenko *et al.*, 2010). Further studies besides the activation of MPK and TGF-/SMADS pathways can explain the phenomena observed here, confirming the formulated hypotheses and bioinformatic inferences. Additionally, it is necessary to verify the expression at the protein level and furthermore, verify the secretion and extracellular activity of these enzymes to corroborate a possible temporal regulation, like the one we propose.

The invasion of the maternal endometrium by EVTs is a key process (He *et al.*, 2017) whose deregulation, seen as hypoinvasiveness or hyperinvasiveness, lead to preeclampsia or trophoblastic neoplasms, respectively (Chakraborty *et al.*, 2002). Being TGF-β a main regulatory factor in trophoblast physiology, including invasion (Pollheimer and Knöfler, 2005), and taking into account the over-expression of this factor in preeclampsia and hydatidiform mole (Pang and Xing, 2003); understanding the molecular mechanism underlying this regulation is important. The possible temporal regulation and the observed dual effects of TGF-β on the expression of MMP9 and uPA, key proteases in the invasive phenotype, are of remarkable interest and they confer an important role to these enzymes in the invasive process of trophoblastic cells, which is highly regulated in a spatial-temporal way (Menkhorst *et al.*, 2016). These results are in agreement with previous reports about the effects of TGF-β in trophoblast and are compatible with the dual and context-dependent TGF-β role in cancer (Ikushima and Miyazono, 2010). Additionally, novel results were obtained, as the temporal regulation of the expression of MMP9 and uPA, involving opposed effects of TGF-β; and remarkably, the influence of serum on the nature and effect of TGF-β on the expression of uPA, a concomitance not previously studied in this model.

CONCLUSIONS

The nature and magnitude of the effects of TGF-β on the expression of uPA and MMP9 in HTR-8/SVneo human immortalized trophoblastic cells depend on the presence of serum, being the culture conditions and stimulation relevant when analyzing the effect of this cytokine.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Belkacemi L, Lash GE, Macdonald-Goodfellow SK, Caldwell JD, Graham CH. Inhibition of human trophoblast invasiveness by high glucose concentrations. J Clin Endocrinol Metab. 2005;90(8):4846-51. Doi:http://dx.doi.org/10.1210/jc.2004-2242

Cerami EG, Gross BE, Demir E, Rodchenkov I, Babur Ö, Anwar N, *et al.* Pathway Commons, a web resource for biological pathway data. Nucleic Acids Res. 2011;39(suppl 1):D685-D690. Doi:http://dx.doi.org/10.1093/nar/gkq1039

Cohen M, Meisser A, Bischof P. Metalloproteinases and Human Placental Invasiveness. Placenta. 2006;27(8):783-793. Doi:http://dx.doi.org/10.1016/j.placenta.2005.08.006

Chakraborty C, Gleeson LM, McKinnon T, Lala PK. Regulation of human trophoblast migration and invasiveness. Can.J. Physiol. Pharmacol 2002;80(2):116-124. Doi:http://dx.doi.org/10.1139/Y02-016

Chen EY, Tan CM, Kou Y, Duan Q, Wang Z, Meirelles GV, *et al.* Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC bioinformatics. 2013;14:128. Doi:http://dx.doi.org/10.1186/1471-2105-14-128

Cheng J-C, Yi Y, Chang H-M, Leung PCK. TGF-β1 up-regulates cadherin-11 expression through Snail: A potential mechanism for human trophoblast cell differentiation. Cell Signal. 2018;43:55-61. Doi:https://doi.org/10.1016/j.cellsig.2017.12.004

Ferretti C, Bruni L, Dangles-Marie V, Pecking AP, Bellet D. Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. Hum Reprod Update. 2007;13(2):121-141. Doi:http://dx.doi.org/10.1093/humupd/dml048

Friedl P, Wolf K. Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer. 2003;3(5):362-374. Doi:http://dx.doi.org/10.1038/nrc1075

Graham CH, Hawley TS, Hawley RC, MacDougall JR, Kerbel RS, Khoo N, *et al.* Establishment and Characterization of First Trimester Human Trophoblast Cells with Extended Lifespan. Exp Cell Res. 1993;206(2):204-211. Doi:http://dx.doi.org/10.1006/excr.1993.1139

He N, van Iperen L, de Jong D, Szuhai K, Helmerhorst FM, van der Westerlaken LAJ, *et al.* Human Extravillous Trophoblasts Penetrate Decidual Veins and Lymphatics before Remodeling

Spiral Arteries during Early Pregnancy. PLOS ONE. 2017;12(1):e0169849. Doi:http://dx.doi.org/10.1371/journal.pone.0169849

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 2007;8(2):R19. Doi:http://dx.doi.org/10.1186/gb-2007-8-2-r19

Huang Z, Li S, Fan W, Ma Q. Transforming growth factor beta1 promotes invasion of human JEG-3 trophoblast cells via TGF-beta/Smad3 signaling pathway. Oncotarget. 2017;8(20):33560-33570. Doi:http://dx.doi.org/10.18632/oncotarget.16826

Ikushima H, Miyazono K. TGFβ signalling: a complex web in cancer progression. Nat Rev Cancer. 2010;10(6):415-424. Doi:http://dx.doi.org/10.1038/nrc2853F

Karmakar S, Das C. Regulation of Trophoblast Invasion by IL-1β and TGF-β1. Am J Of Reprod Immunol. 2002;48(4):210-219. Doi:http://dx.doi.org/10.1034/j.1600-0897.2002.01151.x

Kholodenko BN, Hancock JF, Kolch W. Signalling ballet in space and time. Nat Rev Mol Cell Biol. 2010;11(6):414-426. Doi:http://dx.doi.org/10.1038/nrm2901

Kutmon M, Lotia S, Evelo CT, Pico AR. WikiPathways App for Cytoscape: Making biological pathways amenable to network analysis and visualization. F1000Res. 2014;3:152. Doi:http://dx.doi.org/10.12688/f1000research.4254.2

Lala PK, Graham CH, Lysiak JJ, Khoo NKS, Hamilton GS. TGFβ regulation of trophoblast function. Placenta. 1998;19:149-157. Doi:http://dx.doi.org/10.1016/S0143-4004(98)80012-X

Lala PK, Lee BP, Xu G, Chakraborty C. Human placental trophoblast as an in vitro model for tumor progression. Can.J. Physiol. Pharmacol. 2002;80(2):142-149. Doi:http://dx.doi.org/10.1139/y02-006

Lala PK, Chakraborty C. Factors Regulating Trophoblast Migration and Invasiveness: Possible Derangements Contributing to Pre-eclampsia and Fetal Injury. Placenta. 2003;24(6):575-587. Doi:http://dx.doi.org/10.1016/S0143-4004(03)00063-8

Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Inhibition of Trophoblast Cell Invasion by TGFB1, 2, and 3 Is Associated with a Decrease in Active Proteases. Biol Reprod. 2005;73(2):374-381. Doi:http://dx.doi.org/10.1095/biolreprod.105.040337

Li DQ, Shao ZM. Chapter 19 - Emerging Therapeutic Targets for Cancer Metastasis: From the Perspective of Embryo Implantation. In: Ahmad A, editor. Introduction to Cancer Metastasis. Ciudad: Academic Press; 2017. p. 353-373. Doi:https://doi.org/10.1016/B978-0-12-804003-4.00019-0

Lin HY, Yang Q, Wang HM, Qi JG, Zhang H, Wang HX, et al. Involvement of SMAD4, but not of SMAD2, in transforming growth factor-beta1-induced trophoblast expression of matrix metalloproteinase-2. Front Biosci. 2006;11:637-46. Doi:http://dx.doi.org/10.2741/1823

Liu Y, Fan X, Wang R, Lu X, Dang Y-L, Wang H, et al. Single-cell RNA-seq reveals the diversity of trophoblast subtypes and patterns of differentiation in the human placenta. Cell Res. 2018;28(8):819-832. Doi:10.1038/s41422-018-0066-y

Lunghi L, Ferretti M, Medici S, Biondi C, Vesce F. Control of human trophoblast function. Reprod Biol Endocrinol. 2007;5(1):6. Doi:http://dx.doi.org/10.1186/1477-7827-5-6

Mahmood N, Mihalcioiu C, Rabbani SA. Multifaceted Role of the Urokinase-Type Plasminogen Activator (uPA) and Its Receptor (uPAR): Diagnostic, Prognostic, and Therapeutic Applications. Front Oncol. 2018;8(24). Doi:http://dx.doi.org/10.3389/fonc.2018.00024

Mandl M, Haas J, Bischof P, Nohammer G, Desoye G. Serum-dependent effects of IGF-I and insulin on proliferation and invasion of human first trimester trophoblast cell models. Histochem Cell Biol. 2002;117(5):391-9. Doi:http://dx.doi.org/10.1007/s00418-002-0403-5

Meisser A, Chardonnens D, Campana A, Bischof P. Effects of tumour necrosis factor-β, interleukin-1β, macrophage colony stimulating factor and transforming growth factor β on trophoblastic matrix metalloproteinases. Mol Hum Reprod. 1999;5(3):252-260. Doi:http://dx.doi.org/10.1093/molehr/5.3.252

Menkhorst E, Winship A, Van Sinderen M, Dimitriadis E. Human extravillous trophoblast invasion: intrinsic and extrinsic regulation. Reprod Fertil Dev. 2016;28(4):406-415. Doi:https://doi.org/10.1071/RD14208

Novoa-Herran S, Umaña-Perez A, Canals F, Sanchez-Gomez M. Serum depletion induces changes in protein expression in the trophoblast-derived cell line HTR-8/SVneo. Cell Mol Biol Lett. 2016;21(1):22. Doi:http://dx.doi.org/10.1186/s11658-016-0018-9

Pang Z-J, Xing F-Q. Expression of transforming growth factor-β and insulin-like growth factor in molar and placental tissues. Arch Gynecol Obstet. 2003;269(1):1-4. Doi:10.1007/s00404-002-0379-3

Pardali K, Moustakas A. Actions of TGF-[beta] as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta Rev Cancer. 2007;1775(1):21-62. Doi:http://dx.doi.org/10.1016/j.bbcan.2006.06.004

Pollheimer J, Knöfler M. Signalling pathways regulating the invasive differentiation of human trophoblasts: a review. Placenta. 2005;26:21-30. Doi:http://dx.doi.org/10.1016/j.placenta.2004.11.013

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 2003;13(11):2498-2504. Doi:http://dx.doi.org/10.1101/gr.1239303

Shimonovitz S, Hurwitz A, Barak V, Dushnik M, Adashi EY, Anteby E, *et al*. Cytokine-mediated regulation of type IV collagenase expression and production in human trophoblast cells. J Clin Endocrinol Metab. 1996;81(8):3091-6. Doi:http://dx.doi.org/10.1210/jc.81.8.3091

Staun-Ram E, Goldman S, Gabarin D, Shalev E. Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. Reprod Biol Endocrinol. 2004;2(1):59. Doi:http://dx.doi.org/10.1186/1477-7827-2-59

Turunen SP, Tatti-Bugaeva O, Lehti K. Membrane-type matrix metalloproteases as diverse effectors of cancer progression. Biochim Biophys Acta Mol Cell Res. 2017;1864(11, Part A):1974-1988. Doi:https://doi.org/10.1016/j.bbamcr.2017.04.002

Weber M, Knoefler I, Schleussner E, Markert UR, Fitzgerald JS. HTR8/SVneo Cells Display Trophoblast Progenitor Cell-Like Characteristics Indicative of Self-Renewal, Repopulation Activity, and Expression of "Stemness-"; Associated Transcription Factors. BioMed Res Int. 2013;2013:10. Doi:10.1155/2013/243649

Yanzhen ZUO, Zhihua FU, Yatao HU, Yuhong LI, Qian XU, Dayong SUN, *et al*. Effects of transforming growth factor-*β*1 on the proliferation and invasion of the HTR-8/SVneo cell line. Oncol Lett. 2014;8(5):2187-2192. Doi:http://dx.doi.org/10.3892/ol.2014.2451

Yi Y, Cheng J-C, Klausen C, Leung PCK. TGF-beta 1 inhibits human trophoblast cell invasion by upregulating cyclooxygenase-2. Placenta. 2018;68:44-51. Doi:http://dx.doi.org/10.1016/j.placenta.2018.06.313

Yudate T, Isaka K, Kosugi Y, Koshiishi M, Shiraishi K, Hosaka M, *et al*. Analysis of the mechanism of trophoblast infiltration. Nihon Sanka Fujinka Gakkai Zasshi. 1996;48(3):191-8.

Zhang S-M, Tian F-J, Zeng W-H, Ma X-L, Ren J-B, Lin Y. XCL1-XCR1 pathway promotes trophoblast invasion at maternal-fetal interface by inducing MMP-2/MMP-9 activity. Am J Of Reprod Immunol. . 2018;80(3):e12990. Doi:https://doi.org/10.1111/aji.12990

Zhao M-r, Qiu W, Li Y-x, Zhang Z-b, Li D, Wang Y-l. Dual effect of transforming growth factor *β*1 on cell adhesion and invasion in human placenta trophoblast cells. Reproduction. 2006;132(2):333-341. Doi:http://dx.doi.org/10.1530/rep.1.01112

Zheng Q, Yang Y, Cui X, Zhang D, Liu S, Yan Q. AP1 mediates uPA/uPAR induced FUT4 expression and trophoblast invasion. J Cell Biochem. 2018;119(8):6442-6451. Doi:http://dx.doi.org/10.1002/jcb.26648